Book 4

84

CONTRACTS

SCIENTIFIC ADVISORY BOARD

October 30, 31, November 1, 1974

1003536093

CONTRACTS

The Council for Tobacco Research - U.S.A., Inc.
Scientific Advisory Board
October 30, 31 - November 1, 1974

	Amount	Period
1. Emphysema		
Nims - M.A.	$117,800	1/1/75-12/31/77 (3 years)
2. Human AHH Studies		
23 a) Pike - U.S.C.	$ 63,899	11/1/74-10/31/75
25 b) Kouri - M.A. #2225	$108,406	11/11/74-12/31/75
3. Dosimetry		
15 Oak Ridge - Stokely	$ 94,000	1/1/75-12/31/75
4. Animal Carcinogenesis Model		
2 Whitmire - M.A. #2220 Suspended	$181,560	1/1/75-12/31/75
5. Virus in Chemical Carcinogenesis		
9 a) Jay Levy - U.C.M.C.-S.F.(grant)	$ 60,000	1/1/75-12/31/75
b) M.A. - Breeding	$ 40,000	
6. Engineering		
12 a) Oak Ridge (Lorillard Smoking machine)	$ 15,000	11/1/74-10/31/75
10 b) Process & Instruments	$ 25,000	1/1/75-12/31/75
21 c) P&I - Authorization for Commercial Use		
7. Fractionation		
24 a) Meloy - Patel	$ 14,000	1/1/75-12/31/75
b) Oak Ridge	$150,000	
	~~$85,065~~	
	869,665	

1003536094

1003536095

EMPHYSEMA

1003536096

NIMS - M.A.

PROPOSAL FOR CONTRACT
BETWEEN
THE COUNCIL FOR TOBACCO RESEARCH - USA
AND
MICROBIOLOGICAL ASSOCIATES
FOR
STUDIES WITH A PULMONARY EMPHYSEMA
ANIMAL MODEL SYSTEM.

Date: September 16, 1974

1003536097

RESEARCH CONTRACT PROPOSAL

TITLE: "Studies with a Pulmonary Emphysema Animal Model System."

TO: Council for Tobacco Research
110 East 59th Street
New York, New York 10022

FROM: Microbiological Associates, A Division of Dynasciences Corporation
4733 Bethesda Avenue
Bethesda, Maryland 20014

DATE: September 16, 1974

Vincent L. Ruwet
Vincent Ruwet
Vice President, Contracts and Administration

1003536095

TABLE OF CONTENTS

OBJECTIVES

The ultimate objective would be to have a small mammal with a genetic susceptibility to the dilation of air spaces distal to the bronchiole, clinically defined as emphysema. Such a model system is needed to define the effects of an overlay of air pollutants, dusts and smoking. These effects can be used to show whether dust, air pollution or smoke constitutents overlayed upon the natural genetic susceptibility, would lead to the centrilobular localized form of emphysema commonly occurring in man.

This proposed contract would initiate a breeding colony composed of subfamilies of BALB/c mice. Inbreeding of selected parents would be directed toward high or low incidence of emphysema. Further, a holding colony would be maintained and research directed toward defining the parameters of lung anatomical and physiological changes with age, tryptic enzyme inhibition serum levels and specific serum lung anti-collagen, and anti-elastin directed antibody responses.

INTRODUCTION

Thurlbeck[1], in a review paper, analyzes the difficulties in the differential diagnosis of chronic obstructive lung disease. Approximately 20% of adult human males have chronic bronchitis; apparently two-thirds of this group also have emphysema. It is uncommon to find adult lungs entirely free of emphysema at necropsy and approximately 50% of adults have "significant" defined centrilobular and/or panacinar emphysema at necropsy.

Research into the pathogenesis of emphysema, a chronic pulmonary disease restricted to dilation of air spaces distant to the terminal bronchiole, has been limited by lack of a suitable natural small mammal model of the disease.

A number of artificially induced models of emphysema have been reported. Recently Snider et al[2] reported a centrilobular emphysema in rat lungs, associated with fibrosis, experimentally induced by cadmium chloride aerosol. Krehl[3] induced panlobular emphysema through increasing resistance to bronchial air flow with a ball valve. Tura[4] subjected rats to a forced swim daily for 90 days, Gross[5] introduced papain intratracheally and Giles[6] introduced papain into rat lungs in an aerosol. It has been reported that emphysema is a major cause of disability in racing dogs[7]. Equine pulmonary emphysema, as a result of broncho-pulmonary mold allergy, has been reported[8]. Carlson[9] showed that high doses of 3-methylindole, a natural trytophane metabolite, given

intragastrically, result in emphysematous lesions in goats and cattle. Nettesheim[10] reported panlobular emphysema in hamsters as a result of chromate dust inhalation. Subacute NO_2 induced lesions of rat lungs were noted by Freeman[11], and SO_2 inhalation results in a generalized panlobular emphysema[12]. The relationship between experimentally induced emphysema in cattle, rats or dogs and naturally occurring pulmonary disease in humans is not clear. To date no animal model analogous to the congenital $alpha_1$ trypsin deficiency[13] associated with some human panlobular emphysema has been shown, although Chan[14] has defined $alpha_1$ antitrypsin inhibitors in different strains of mice.

Dr. Louise Rabstein, a veterinary pathologist at Microbiological Associates, undertook a review of slides from over 900 mice of nine different strains between 3 and 33 months of age from the long term holding colonies at Walkersville, Maryland. The most consistent finding of emphysema was in a pedigree BALB/c colony which had been maintained since 1968. All breeding within the colony was brother to sister matings. Rather than maintaining the pedigree as a single line of descendants, a number of sub-strains were developed. In 1971, this was reduced to seven separate sub-strain families (Families G, L, S, X, Y, 1 and 2). The studies initiated by Dr. Rabstein have been continued by Dr. Bernard Sass, Veterinary Pathologist.

Mice in the Pedigreed BALB/c colony are allowed to live their maximum life span. They are examined weekly for evidence of palpable enlargements. All moribund animals are sacrificed and a complete necropsy performed. Dead animals, if not decomposed, are also necropsied. Following processing of tissues by the histology laboratory, the slides are microscopically reviewed and a diagnosis rendered blindly (without knowledge of family).

The data presented are based upon the microscopic review of pulmonary tissue from mice of the 7 sub-families comprising this pedigreed BALB/c colony. Animals with lesions of pulmonary emphysema were classified, using a scale of severity of lesions, from negative to 4+, based on a subjective rating system devised by Dr. Rabstein. Her criteria for classification were:

Negative - no perceptible overdistention

1 plus - minimal overdistention in minimal number of lobes.

2 plus - slight overdistention in more than 1 lobe.

3 plus - mild to moderate overdistention in 1 or more lobes.

4 plus - areas of severe overdistention and/or bullae formation in one or more lobes.

Fig. 1 illustrates representative examples of each classification.

In 1973 Dr. Rabstein reported familial variance of spontaneously occurring generalized emphysema in the old BALB/c mice (greater than 18 months)*. Dr. Robert Kovatch and other consultant pathologists reviewed and concurred in these findings. The histomorphology of the emphysema in mice fits most nearly into the panacinar (diffuse vesicular) or alveolar category. The more prominent lesions in BALB/c mice occur toward the periphery of the lung lobes. Centrilobular emphysema as described in human lungs has not been observed.

Large numbers of mice have been studied since that time by Dr. Bernard Sass, and the differing familial incidences have been reconfirmed,lending credence to the possibility of developing a congenital emphysema model for experimental research purposes.

* Report, L. Rabstein to J. Kreisher, C.T.R., Feb. 1973.

METHODS

Gross and Microscopic Examinations

Mice are sacrificed by placing them in a chamber containing dry ice. When anaesthetized, they are killed by exsanguination from the brachial artery. The heart and lungs are removed from the thoracic cavity in toto. A complete gross examination is also performed. Animals whose lungs are to be inflated are handled as above, but in addition, approximately 1.5 cc of 10% neutral buffered formalin is instilled intratracheally at a pressure of approximately 10 cm water prior to removal from the thoracic cavity. A 100 ml burette is used as a reservoir and plastic tubing with an attached blunt 18 ga needle serves as a canula. The trachea is clamped off and tied following perfusion. The heart and lungs are then immersed in 10% neutral formalin overnight. The entire heart and lungs are processed, embedded in paraffin and 6 micron sections are cut and stained with Hematoxylin and Eosin.

Alpha$_1$ Antitrypsin

Blood samples are collected by the orbital bleeding technique using .280 ml capillary tubes. When the blood is clotted, the tubes are centrifuged at 3000 rpm for 15 minutes in a refrigerated centrifuge. The resultant serum is frozen and submitted to Dr. D. Michaeli, Dept. of Biochemistry, University of California Medical School, San Francisco. The method of analysis for alpha$_1$ antitrypsin is the enzymatic method of Sachar et al (1955).

Physiology

Functional residual capacity will be measured in the anaesthetized mouse by the method of Watanabe and Aviado (in press). Tidal volume, pulmonary resistance and pulmonary compliance measurements will be obtained using methods outlined by Policek et al (1967) and Ito and Aviado (1968).

RESULTS TO DATE

Incidence and Severity

Table A-1 is a tabulation of the incidence and severity of emphysema in 567 individual mice from the 7 sub-families. The percentage of individuals with 3+ or 4+ emphysema ranged from a low of 38% for Family 1 to a high of 61% for Family G. The average of all families was 52%. Table A-2 shows the mean age of mice in each family with the different degrees of severity of pulmonary emphysema.

On the basis of these findings, Families S, X and Y were excluded from further analysis of results, leaving 3 families (G, L and 2) with a higher than average incidence of severe (3+ or 4+) emphysema and one (Family 1) with a lower than average incidence. Family 1 was retained as a low incidence control sub-strain. The comparative difference in incidence and severity of emphysema between the high and low incidence families is shown in Fig. 2. There is more of the severe (3+ and 4+) emphysema in the high incidence families. In Family 1 (low incidence), the trend is toward the milder forms of emphysema.

Age Relationships

Figure 3 and Table A-2 shows that the severity of emphysema, in general, increases with advancing age. Further, the mean age of occurrence of emphysema is generally earlier in the high incidence families.

Sex Differences

The incidence and severity of emphysema by sex was determined and is shown in Table B and Fig. 4. This was done because more female mice were kept to old age. Emphysema of all levels of severity was generally equal in distribution between the two sexes.

Effect of Method of Fixation

Early in this study, the degree of emphysema was established based on uninflated, formalin-fixed lung sections. Later, it was suggested the accuracy of diagnosis would be improved if the lungs were fixed in an inflated state. After that time, most of the lungs were inflated. Table C is a tabulation of the incidence and severity of emphysema in inflated and uninflated lungs. The comparison is summarized in Fig. 5. The incidence of all degrees of severity of emphysema in uninflated lungs is comparable with that of inflated lungs. This suggests that, although there may be an advantage to examining the inflated specimen, the data from uninflated lungs is acceptable for computations of incidence.

Age Association

The frequency of emphysema in mice of various ages is shown in Table D. Frequency was calculated for negative, 1+ and 2+ and for 3+ and 4+. The ages of greatest incidence for the negative, 1+ and 2+ group was from 16 through 24 months of age (79% of individuals).

The 3+ and 4+ group was 19 through 27 months of age (84% of individuals). In both groups, over 60% were in the 19 through 24 month age group. This indicates that 3+ or 4+ emphysema is infrequently found in this strain of mice prior to 18 months of age.

The incidence of severe (3+ or 4+) emphysema in the high (Families G, L and 2) and low (Family 1) incidence families, at various age groups, is compared in Table E and Fig. 6. Except for the young and the very old age groups, where numbers of observations are too small to be valid, the frequency of occurrence of severe emphysema is markedly greater in the high incidence families, involving over 50% of the mice examined at ages above 19 months.

Associated Lung Disease Incidence

Table F, which lists the frequency of observance of lesions other than emphysema, indicates that the age at which emphysema is most often seen coincides with the age of greatest frequency of other findings. The frequency of findings of the various categories of pulmonary and non pulmonary lesions is shown in Table G.

The data in Tables F and G for the high incidence families (G, L and 2) were combined and compared to the low family, based on the numbers of mice with lesions divided by the total number observed for each age group. The results are seen in Table H and Figures 7 and 8.

For pulmonary lesions (Fig. 7) the high incidence families had greater frequency at all age groups except the 19-21 month group, where the frequency is equal. The frequency of non-pulmonary lesions (Fig. 8) was more nearly equal in the two groups for those ages (16-24 months) in which there were significant numbers of mice to compare.

The comparative frequency of the various types of lung lesions in the 4 families (Fig. 9) indicates that Family 1 had a small but consistently lower frequency of lesions of all types except reticuloendothelial neoplasms. Here, the incidence was essentially the same for all families. Family L, a high incidence family, had frequencies for all lesions similar to low incidence family 1. This interesting observation will be closely followed in continuing genetic studies.

Table I demonstrates the general comparability of the four families when the 3+ and 4+ emphysema is matched by age with other pulmonary and non pulmonary lesions. This emphasizes that, in general, all families had the same experience; that of having emphysema at approximately the same age as other lesions. These data suggest that the emphysema may well be related to the occurrence of other pulmonary lesions as well as space-taking lesions of other organ systems. The fact that, in spite of this,

there are families characterized by low and high emphysema incidence, argues for factors other than concurrent lesions having a part in the frequency of occurrence of emphysema.

Table J is a compilation, by family, of those mice with various pulmonary lesions and associated mild (negative to 2+) or severe (3+ and 4+) emphysema. Based on a very small sample, the severe emphysema is, in general, more frequently seen in animals with other pulmonary lesions than is the mild emphysema. Family 1 is the exception; lung tumors and other lung lesions were approximately the same in the neg to 2+ and the 3+ and 4+ groups. Family 2 had the highest incidence of concurrent lung lesions with 3+ and 4+ emphysema as compared with the neg to 2+ incidence. (Family G, 2:1; Family L, 2:1; Family 2, 9:1; Family 1, 1:1.)

The comparative frequency of severe emphysema with concurrent lung lesions in the high and low incidence families is seen in Fig. 10. Although based on small numbers of animals for each age group in Family 1, there is a consistent pattern of greater frequency in the high incidence families. As with the other tabulations, the increase in occurrence with increasing age is obvious for both groups.

Alpha$_1$ Trypsin Inhibition Assay

In collaboration with Dr. Dov Michaeli, of the University of California at San Francisco, preliminary studies of alpha$_1$ antitrypsin levels have been carried out on young mice being held for future disease studies. Individual and sex associated differences were observed in the various families. Males had significantly higher alpha$_1$ trypsin inhibitor as is shown in Fig. 11. In initial studies of a limited number of emphysematous mice significant blood hemolysis occurred, but no correlation was evident between percentage of inhibition and emphysema expression. This alpha$_1$ trypsin inhibitor series is being repeated in a larger number of animals.

Pedigree Definition

When individual mice with 3+ or 4+ emphysema are identified on the pedigree chart of a family, it becomes possible to separate, within the family, those breeding lines which have the greatest or least numbers of individuals with emphysema (Fig. 12).

Mice from this pedigreed BALB/c colony having 3+ and 4+ emphysema, reflect an average incidence of 52% at 21 months of age, with highest incidence (61%) in Family G. Family 1, at the other extreme, had an incidence of 38% (Table A). This suggests there might be a genetic determinant of predisposition for emphysema. The fact that the incidence of concurrent pulmonary and non-pulmonary pathologic lesions displays less than the above variation between the high and low incidence families (Table F) further suggests a genetic factor.

DISCUSSION OF RESULTS TO DATE

The mice from which these data were developed were kept for another purpose; the definition of the natural history of neoplasia throughout their life span. This precluded doing serial sacrifices and leaves unanswered the question of the rate at which emphysema develops, the progression of the lesion and the earliest age at which a 50% incidence of emphysema can be predicted. It also precluded concurrent physiological and biochemical studies such as are included in the following proposal. Studies of this nature would require establishment of a colony specifically for utilization by CTR, with the concurrent investigation of specifically associated causative factors.

EXPERIMENTAL DESIGN AND PROCEDURES

Breeding and Holding Colony

Microbiological Associates proposes to initiate a two part program to define the genetics of emphysema in mice. First a production (breeding) colony of pedigreed BALB/c mice will be maintained, utilizing breeders selected from those parental lines in which the expression of emphysema is high or low. Second a holding (research) colony for studying age at incidence of emphysema onset and for physiology, biochemical and immunological testing in the three high incidence pulmonary emphysema families and the one low incidence family.

The production will be based on the numbers of breeding and holding mice that can be maintained on 15 mouse cage racks. It is estimated that this will, in time, provide a constant yield of approximately 100 mice, 24 month old, per month. Actual numbers at each age increment will depend upon the number of mice sacrificed for experimental studies prior to 24 months of age.

Four pedigree families; one low incidence line (Family 1) and 3 high incidence lines (Families G, L and 2) will be maintained. Accurate pedigrees will be kept on each family, to include positive identification of all breeder mice by ear and/or toe marking. Holding colony mice will be separated by sex and each age will be identified by cage

cards. Each mouse will be identified by its sub-family and parents. At the end of their active breeding life, the production colony mice will be added to the holding colony population. This will provide an added number of mice for experimental purposes.

Once the holding colony is established, breeding schedules will be set to assure a steady number of weanling age mice. Continued accumulation of data on which to base the breeding program will be carried out during the transfer of effort from NCI contract #NCI CP-33248 to CTR. In the second year, mice 12 months of age will become available for sampling to determine pulmonary physiological and biochemical parameters.

Sampling for serologic determination of the murine virus profile of the colony will be scheduled at regular intervals, to assure that the disease status of the colony is always known. There will be a similar bacteriological monitoring of the mice.

When a definitive baseline of physiological, biochemical and pathological parameters has been established, it will become possible to conduct induction studies utilizing a variety of materials. Such induction studies might well lead to the changing of the panlobular emphysema to the centrilobular type most frequently seen in humans.

As it becomes possible to predict which individual mice will develop severe emphysema, more definitive genetic studies can be planned, utilizing f_1 hybrids and back crosses to identify gene loci of the hereditary factors contributory to the development of severe emphysema.

LUNG PHYSIOLOGY, BIOCHEMISTY AND PATHOLOGY

A collaborative study with consultant Dr. D. Aviado of the Dept. of Pharmocology, Univ. of Penna. will be undertaken to define those physiological parameters which might be predictive of the disease status of living mice. Groups of animals will be withdrawn from the holding colony at different ages for study. Male and female mice from each family will be killed at 3 month intervals to establish a histologic diagnosis of emphysema. The parameters of α_1 antitrypsin inhibitor level, specific serum lung collogen antibodies, functional residual capacity, pulmonary resistance and compliance, ventilatory response and blood gas analyses will be determined at regular 3 mo. intervals prior to sacrifice.

All animals on this Project that are sacrificed or die will be subjected to a complete gross and microscopic pathology examination. Results of this examination will be recorded and entered into a computerized storage. This will permit correlation of data with experimental results, physiological and biochemical testing procedures, and other ancillary data. Programs will be formulated to permit rapid retrieval and analysis of data to provide those correlations required to interpret the results of experimental protocols. The data input will include pedigree records to assist in establishing the genetic history of individual mice.

FACILITIES

The room to be set aside for this project is located in Bldg. #3, Ballenger Creek. It will provide a separate room within a disease barrier sustained colony building. This room meets or exceeds the requirements of the Guide for the Care and Use of Laboratory Animals, DHEW Publication No. (NIH) 73-23, revised 1972. The space is also accredited by the American Association for the Accreditation of Laboratory Animal Care. This gives maximum assurance against the inadvertent introduction of disease into the colony.

A temperature of $74^{o} \pm 4^{o}F$ will be maintained in the animal room. There will be 8-10 changes of air per hour with 100% air exchange (no recirculation).

Drinking water will be chlorinated to 10-12 PPM as an adjunct to control of Salmonella. All feed entering the building will be pasteurized and all bedding sterilized by steam autoclave.

Cage units (cage, lid and watering device) will be changed weekly. Before re-use they will be sanitized by passage through a 3 cycle cage wash machine. Cage wastes will be sealed in plastic bags for removal from the animal room.

Personnel assigned as animal caretakers will be utilized exclusively for this project. They will pass through a personnel entry lock each time they enter the

building. Here, they will remove their street clothes, shower, then change into a clean working uniform. This will include shoes to be worn only in the animal room, a disposable face mask and a disposable head cover.

REFERENCES

1. Thurlbeck, W.M., et. al. Chronic Obstructive Lung Disease. Med. 49:81-145 (1970).

2. Snider, G.L., Hayes, J.A., Karthy, A.L. & Lewis, G.P. Centrilobular emphysema experimentally induced by cadmium chloride aerosol. Amer. Rev. Resp. Disease 108:40-48 (1973).

3. Krehl, V.E. The experimental production of pulmonary emphysema, A preliminary report. Amer. Rev. Resp. Disease 80:158-167 (1959).

4. Tura, S. Pulmonary emphysema and polycythemia induced in rats by forced swimming. Proc. Soc. Exp. Biol. Med. 103:713-715 (1960).

5. Gross, P., et al. Experimental emphysema: Its production with papain in normal and silicotic rats. Arch. Environ. Health 11:50-58 (1965).

6. Giles, R.E. Production of emphysema like conditions in rats by administration of papain aerosol. Proc. Soc. Expt. Biol. & Med. 134:157-162 (1970).

7. Pugh, P.S. Greyhound Racing Association (personal communication).

8. Eyre, P. Equine Pulmonary Emphysema - A Bronchopulmonary Mould Allergy. Vet. Rec. 91:134-140 (Aug. 1972).

9. Carlson, J.R., et al. Induction of pulmonary edema and emphysema in cattle and goats with 3-methylindole. Science 176:298-299 (21 Apr 1972).

10. Nettesheim, P., (personal communication).

11. Freeman, G., et al. Subacute NO_2 induced lesions of rat lung. Arch. of Env. Health (Chicago) 18:609-612 (April 1969).

12. Goldring, I.P., et al. Pulmonary effects of SO_2 in hamsters II. Combinations with emphysema. Arch. of Env. Health (Chicago) 21:32-37 (June 1970).

13. Eriksson, S. Pulmonary emphysema and $Alpha_1$ antigen defining. Acta Med. Scand. 175:197-205 (1964).

14. Chan, S.K. $Alpha_1$ antitrypsin in sera of inbred mice. Arch. Environ. Health. 27:271-272 (Oct. 1973).

15. Sachar, L., et al. An enzymatic method for the determination of $Alpha_1$ antitrypsin. Proc. Soc. Exptl. Biol. and Med. 90:327 (1955).

16. Aviado, D.M. and Watanabe, T. Functional and Biochemical effects on the lung following inhalation of cigarette smoke. I-High and low nicotine cigarettes in mice. Submitted to Toxicol. Appl. Pharmacol.

17. Palecek, F., et al. Emphysema in immature rats - a condition produced by tracheal constriction and papain. Arch. Env. Health 15:332-342, (1967).

18. Ito, H. and Aviado, D. Pulmonary emphysema and cigarette smoke - Experimental induction and use of Bronchodilators in rats. Arch. Env. Health 16:865-870.

BUDGET - FIRST YEAR

A.	Direct Labor (Schedule A)	$22,770
B.	Overhead (115% of A)	26,186
C.	Other Direct Costs (Schedule B)	21,500
D.	Travel	1,500
E.	Total Before G & A	71,956
F.	General and Administrative (16% of E)	11,513
G.	Overtime Premium	48
H.	Total Cost	83,517
I.	Fixed Fee	9,283
J.	Total Before Equipment & Facilities Rearrangement	92,800
K.	Facilities Rearrangements	5,000
L.	Equipment (Schedule C)	20,000
M.	Total Price	$117,800

R: REDACTED MATERIAL

SCHEDULE A

DIRECT LABOR:

Name	Function	Time on Project	Total Hours*	Hour	$
R. Nims	Project Director	15%	289	REDACTED	
B. Sass	Veterinary Pathologist	10%	193	REDACTED	
B. Getzandanner	Histology Technician	10%	193	REDACTED	
W. Athey	Animal Technologist	10%	193	REDACTED	
Vacancy (to start last 3rd of year)	Technician	100%	642	REDACTED	
J. Disney(to start 4th quarter)	Animal Caretaker	100%	482	REDACTED	
P. Lee	Animal Caretaker	100%	1,926	REDACTED	
M. Haven	Computer Programmer	10%	193	REDACTED	
P. Gradwell	Research Clerk	10%	193	REDACTED	

* Less 7.4%

Total Hours: 4,304

Total Direct Labor: 22,107

Plus 6% Merit Raise (3% for 6 months) 663

TOTAL DIRECT LABOR: $22,770

1003536124

SCHEDULE B

OTHER DIRECT COSTS:

Cages, feed and bedding	$13,700
General Supplies	2,300
Computer rental	2,000
Consultant	3,500
TOTAL OTHER DIRECT COSTS:	$21,500

SCHEDULE C

Equipment for physiological studies (force resistant capacity, resistance, compliance, responsiveness to low O_2):	$20,000

CURRICULUM VITAE FOR PROFESSIONAL STAFF

A. Robert M. Nims, D.V.M.

B. Bernard Sass, M.S., V.M.D.

C. Wilbur L. Athey, B.S.

1003536126

R: REDACTED MATERIAL

Soc. Sec. No[redacted]

CURRICULUM VITAE - ROBERT M. NIMS

BIRTH: REDACTED

EDUCATION: 1956 Postgraduate Training (Surgery)
School of Veterinary Medicine
University of Pennsylvania, Philadelphia
1944 D. V. M., Iowa State University, Ames
1941 Pre-Veterinary Medicine
Oklahoma State University, Stillwater

PROFESSIONAL AFFILIATIONS:

REDACTED

PRESENT POSITION: 1970 - present

REDACTED

POSITION DESCRIPTION: REDACTED

PRIOR EXPERIENCE: 1968 - 1970

REDACTED

1967 - 1968

REDACTED

1964 - 1967

REDACTED

1946 - 1963

1944 - 1945

REDACTED

1003536127

PUBLICATIONS - ROBERT M. NIMS

Glyceride Content of Human and Canine Red Blood Cells. Vacca, J.B., Waring, P.P., and Nims, R.M. Proceedings of the Society for Exp. Biol. and Med., 105: 100-102, 1960.

Human Body Volumeter Based on Water Displacement. Allen, T.H., Krzywicki, H.J., Worth, W.S., and Nims, R.M. U.S. Army Medical Research and Nutrition Laboratory Report 250, 1960.

Canine Mammary Adenocarcinoma with Metastasis to Bone. Nims, R.M., Dean, E.E., and Geil, R.G. A Case Report, Journal American Veterinary Med. Assoc., 138: 2: 87-89, 1961.

Studies in Segmental Replacement of the Thoracic Trachea. Aronstam, E. M., Nims, R.M., and Winn, D.F., Jr. Journal of Surgical Research, 1: 2: 108-110, 1961.

Fabrication of a Canine Respiratory Face Mask. Nims, R.M., and Worth, W.S. Jour. App. Physiol. 16: 1139-1140, 1961.

Embolectomy in the Dog. Nims, R.M. J.A.V.M.A., 140: 618-672, 1962.

Experimental Dislocation of the Femur, Delivered at Orthopedic Section Meeting, AMA, American Hotel, Miami Beach, Florida. Travis, L.O., Nims, R.M., Haupt, E.C., Omar, G.C., and Arnold, R.A., 1963.

Cervical Ganglioneuroma in a Dog. Ferrell, J.J., Hunt, R.D., and Nims, R.M., J.A.V.M.A., 144: 508-512, 1964.

Ehrlichia canis--The Causative Agent of a Hemorrhagic Disease of Dogs? Huxsoll, D.L., Hildebrandt, P.K., Nims, R.M., Ferguson, J.A., and Walker, J.S. Vet. Rec., 85: 587, 1969.

Clinical and Clinicopathologic Findings in Tropical Canine Pancytopenia. Walker, J.S., Rundquist, J.D., Taylor, R., Wilson, B.L., Andrews, M.R., Barck, J., Huxsoll, D.L., Hildebrandt, P.K., Hogge, A.L., and Nims, R. M. J.A.V.M.A., 157: 43-55, 1970.

Experimental Ehrlichiosis in Young Beagle Dogs. Hildebrandt, P.K., Huxsoll, D.L., and Nims, R.M. Fed. Proc., 29: 754, 1970 (Abstract).

The Pathology of Canine Tropical Pancytopenia. Hildebrandt, P.K., Huxsoll, D.L., Nims, R.M., and Walker, J.S. Lab. Invest., 22: 500, 1970 (Abstract).

Epizootiology of Tropical Canine Pancytopenia. Huxsoll, D.L., Hildebrandt, P.K., Nims, R.M., Amyx, H.L., and Ferguson, J.A. Journal of Wildlife Diseases, 6: 220-225, 1970.

Tropical Canine Pancytopenia.. Huxsoll, D.L., Hildebrandt, P.K., Nims, R. M., and Walkers, J.S. J.A.V.M.A., 157: 1627-1632, 1970.

PUBLICATIONS (Cont.)
R.M. NIMS

Epizootiology of Tropical Canine Pancytopenia in Southeast Asia. Nims, R.M., Ferguson, J.A., Walker, J.L., Hildebrandt, P.K., Huxsoll, D.L., Reardon, M.J., Varley, J.E., Kolaja, G.J., Watson, W.T., Shroyer, E. L., Elwell, P.A., Vacura, G.W. J.A.V.M.A., 158: 53-63, 1971.

Development of Hypergammaglobulinemia in Tropical Canine Pancytopenia. Burghen, G.A., Beisel, W.R., Walker, J.S., Nims, R.M., Huxsoll, D.L., and Hildebrandt, P.K. Am. J. Vet. Res., 32: 749-756, 1971.

Tropical Canine Pancytopenia. Huxsoll, D.L., Hildebrandt, P.K., Nims, R. M., and Walker, J.S. in Kirk: Current Veterinary Therapy-IV, Pub., Saunders Co., 677-679, 1971.

Laboratory Studies of Tropical Canine Pancytopenia. Huxsoll, D.L., Amyx, H. L., Hemelt, I.E., Hildebrandt, P.K., Nims, R.M., and Gochenour, W.S., Jr. Exper. Parasit, 31: 53-59, 1972.

An Improved Method for Enumeration of X-C Cell Assay for Murine Leukemia Virus. Spahn, G.J., Nims, R.M., Peters, R.L., and Kenyon, K. Applied Micro., 25: 149-150, 1973 (January).

Production of Hyperimmune Serum With Mature Rabbits. Nims, R.M., and Reeder, D.J. Lab. Animal Science, 23: 391-396, 1973 (June).

Pathology of Canine Ehrlichiosis (Tropical Canine Pancytopenia). Hildebrandt, P.K., Huxsoll, D.L., Walker, J.S., Nims, R.M., Taylor, R., and Andrews, M.A. Amer. Jour. Vet. Res. 34: 1309-1320, Oct. 1973.

CURRICULUM VITAE

NAME: Bernard (NMI) Sass

ADDRESS: REDACTED

PLACE OF BIRTH: REDACTED

DATE OF BIRTH:

EDUCATION:

New Brunswick High School		cademic degree
Rutgers University	1952-56	B.S. (Poultry Sci.)
Univ. of Illinois	1956-57	Nutrition-Bio-chemistry research assistantship
Univ. of Pennsylvania	1957-61	Doctor of Veterinary Medicine (V.M.D.)
Univ. of Maryland	At present	M.S. Microbiology (should be received 1973)

MILITARY SERVICE:

REDACTED

EXPERIENCE: 1963

1963-66 REDACTED

1966-1973

REDACTED

1974-present

PUBLICATIONS:

Messersmith, R.E., Sass, B., Berger, H., Gale, G.O. Safety and Tissue Residue Evaluations in Swine Fed Rations Containing Chlortetracycline, Sulfamethazine and Penicillin. JAVMA, 151, no. 6 (Sept. 15, 1967) pp 716-724.

Albert, T.F., McKinstry, D., Sass, B., Cason, J.L. Rectal Passage of a Duodenal Cannula in a Young Bull. Mod. Vet. Pract., Nov. 1968, p. 48.

McKinstry, D.M., Sass, B., Cason, J.L. Albert T.F. Arteriosclerosis in Forestomach-Bypass Calves. J. of Dairy Sci., Vol. 52 No. 2 (Feb. 1969) pp. 273-276.

Mohanty S.B., Lillie, M.G., Albert, T.F. Sass. B. Experimental Exposure of Calves to a Bovine Rhinovirus. Am. J. Vet. Res., Vol. 30, no. 7 (July, 1969) pp. 1105-1111.

Sass, B., Albert, T.F. A case of Eisenmenger Complex in a Calf. Cornell Vet., Vol. LX, no. 1 (Jan. 1970).

Sass, B. Equine Strongylosis Threatens Horse Population. The Maryland Horse, Vol. 35, no. 4 (April, 1969) pp. 64-65.

Shillinger, R.B., Sass, B., Virts, H.A. An Apparent Outbreak of Botulism in Feedlot Cattle. Maryland Veterinarian, Vol. 12, no. 1 (Feb. 1970).

Sass, B. Perforating Gastric Ulcer Associated with Lead Poisoning in a Dog. JAVMA, Vol. 157, no. 1 (July 1, 1970) pp. 76-78.

Hemken, R.W., Vandersall, J.H., Sass, B.. Hibbs, J.W. Goitrogenic Effects of a Corn Silage-Soybean Meal Supplemented Ration. J. of Dairy Science, Vol. 54, no. 1 (Jan., 1971) pp. 85-88.

Mallack, J., Sass, B., Ludlum, K.W. Dirofilaria immitis in Hunting Dogs from an Area in Maryland. JAVMA, Vol. 159, no. 2 (July 15, 1971) pp. 177-179.

Sass, B., Ludlam. K.W., Mallack, J. Response by Practicing Veterinarians to a Questionnaire on Dog Heartworm in Maryland. Southern Veterinarian, Vol. 9, no. 3 (May-June, 1972) pp. 14-15.

Sass, B. Hatziolos, B.C.. Hayes, J.E. Probable Cadmium Poisoning in a Group of Ponies. Vet. Med., Vol. 67, no. 7 (July, 1972) pp. 745-746.

Sass, B. Bovine Herpes Virus DN 599--Characterization of the Agent by Immunofluorescence. M.S. Thesis, Graduate School, University of Maryland, College Park, Md.

Sass, B., Mohanty, S.B. and Hetrick, F.M. Bovine Herpes Virus DN599 in Characterization of the agent by Immunofluorescence. Am. J. of Veterinary Research.(In press)

McKinstrey, D.M., Carson, J.L., Albert, T.F. and Sass, B. Observations on the Health and Performance of Forestomach By-Pass and Control Calves Fed a Milk Replaced Diet - Submitted to Journal Airy Science.

R: REDACTED MATERIAL

CURRICULUM VITAE

NAME: Wilbur L. Athey

ADDRESS:

DATE OF BIRTH:

PLACE OF BIRTH: REDACTED

EDUCATION: High School - Flintstone High School, Flintstone, Md.
B.S. Agriculture - University of Maryland, College Park, Md.
1954

MILITARY SERVICE:

REDACTED

REDACTED

EXPERIENCE:
1942-1944 Operated 180 Acre General Farm
1946-1950
1954-1955
1955-1958 REDACTED
1958-1962
July 1962 - present REDACTED

ORGANIZATIONS:

REDACTED

1003536432

TABLE A.

1. Incidence and Severity of Pulmonary Emphysema, by Family

Family	Severity of Emphysema												
	Neg.		1+		2+		3+		4+		Total	Combined 3 & 4+	
	No.[a]	%[b]	No.	%	No.	%	No.	%	No.	%		No.	%
G	2	1	8	8	28	29	42	44	17	18	97	59	61
L	2	2	7	9	26	32	36	44	10	12	81	46	57
S	3	2	15	12	36	29	49	40	20	16	123	69	56
X	2	5	0	-	21	51	12	29	6	15	41	18	44
Y	2	3	11	18	24	39	22	36	2	3	61	24	39
1	10	8	16	19	26	31	26	31	6	7	84	32	38
2	2	2	12	15	19	23	31	40	16	20	80	47	59
TOTAL	23	4	69	12	180	32	218	38	77	14	567	295	52

a - Number of mice in group.
b - % of total mice observed in each family.

2. Mean Age of Occurrence of Pulmonary Emphysema, by Family

Family	Severity of Emphysema					
	Neg.	1+	2+	3+	4+	Combined 3 & 4+
G	21.0[a]	19.3	20.8	21.7	20.0	21.4
L	8.5	19.3	19.7	20.6	21.0	20.7
S	18.7	23.9	20.3	21.7	21.8	21.7
X	14.5	-	19.9	19.5	19.7	19.6
Y	18.5	17.9	20.3	20.6	21.5	20.7
1	18.4	19.5	20.3	21.7	20.0	21.4
2	12.5	17.2	19.2	21.3	21.1	21.2
ALL FAMILIES	17.0	19.8	20.1	21.2	20.8	21.0

a - Mean age (Months) of all mice in group.

TABLE B.

Comparative Incidence and Severity of Emphysema in Male and Female Mice

Family	Not Rated		Neg.		1+		2+		3+		4+		Total Mice
	No.[a]	%[b]	No.	%	No.	%	No.	%	No.	%	No.	%	
MALES													
G	0	-	0	-	2	6	8	26	15	48	6	19	31
L	5	13	2	5	1	3	9	24	18	47	3	8	38
2	1	3	0	-	8	22	12	32	11	30	5	14	37
1	4	11	5	14	5	14	10	28	11	31	1	3	36
TOTAL	10	7	7	5	16	11	39	27	55	39	15	11	142
FEMALES													
G	3	4	2	3	6	9	19	28	27	40	11	16	68
L	1	2	0	-	6	12	17	35	18	37	7	14	49
2	3	6	2	4	4	8	7	15	21	44	11	23	48
1	1	2	3	6	10	20	16	32	15	30	5	10	50
TOTAL	8	4	7	3	26	12	59	27	81	38	34	16	215

a - Number of mice observed

b - Percent of total mice observed in each family

TABLE C.

Comparative Incidence and Severity of Emphysema in Inflated and Uninflated Mouse Lung Specimens

Family	Status	Not Rated		Neg.		1+		2+		3+		4+		Total Mice
		No.[a]	%[b]	No.	%	No.	%	No	%	No.	%	No	%	
G	Inf.	1	4	0	-	3	13	6	25	10	42	4	17	24
	Uninf.	2	3	2	3	5	7	21	28	32	43	13	17	75
L	Inf.	0	-	0	-	1	6	6	33	10	56	1	6	18
	Uninf.	6	9	2	3	6	9	20	29	26	38	4	13	69
2	Inf.	2	7	1	4	7	25	3	11	10	36	5	18	28
	Uninf.	2	4	1	2	5	9	16	28	22	39	11	19	57
1	Inf.	0	-	1	5	4	18	7	32	9	41	1	5	22
	Uninf.	5	7	9	13	12	18	19	28	17	25	5	7	67
TOTAL	Inf.	3	3	2	2	15	16	22	24	39	42	11	12	92
	Uninf.	15	6	14	5	28	10	76	28	97	36	38	14	268

a - Number of mice observed

b - Percent of total mice observed in each family

1003536135

TABLE D.

Frequency of Mild (Negative to 2+) and Severe (3+ and 4+) Emphysema in Various Age Groups of Mice

Family	Age (Months)																Total Mice
	< 10		10-12		13-15		16-18		19-21		22-24		25-27		28 & >		
	No.[a]	%[b]	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
Mice with 1+, 2+ or no Emphysema																	
G	2	5	1	3	0	-	6	15	15	38	11	28	5	13	0	-	40
L	5	12	1	2	0	-	4	10	19	46	8	20	3	7	1	2	41
2	2	5	1	3	3	8	11	30	12	32	5	14	3	8	0	-	37
1	2	4	2	4	2	4	10	18	19	35	16	29	4	7	0	-	55
Total	11	6	5	3	5	3	31	18	65	38	40	23	15	9	1	1	173
Mice with 3+ and 4+ Emphysema																	
G	1	2	0	-	1	2	2	3	19	33	22	38	11	19	2	3	58
L	2	4	0	-	1	2	4	9	18	39	15	33	5	11	1	2	46
2	0	-	1	2	4	9	5	11	11	23	17	36	9	19	0	-	47
1	1	3	1	3	1	3	2	6	9	28	11	34	6	19	1	3	32
Total	4	2	2	1	7	4	13	7	57	31	65	36	31	17	4	2	183

a - Number of mice observed
b - Percent of total mice observed in each family

TABLE E.

Frequency of Severe (3+ and 4+) Emphysema in High and Low Incidence Families, by Age Group

Family	Age (Months)															
	< 10		10-12		13-15		16-18		19-21		22-24		25-27		≥ 28	
	Freq.[a]	%	Freq.	%	Freq.	%	Freq.	%	Freq.	%	Freq.	%	Freq.	%	Freq.	%
G	1/3	33	0/1	-	1/1	100	2/8	25	19/34	56	22/33	67	11/16	69	2/2	100
L	2/7	29	0/1	-	1/1	100	4/8	50	18/37	49	15/23	65	5/8	63	1/2	50
2	0/2	-	1/2	50	4/7	57	5/16	31	11/23	48	17/22	77	9/12	75	0/0	-
Total Fam. G, L & 2) (High Inc.)	3/12	25	1/4	25	6/9	67	11/32	34	48/94	51	54/78	69	25/36	69	3/4	75
Family 1 (Low Inc.)	1/3	33	1/3	33	1/3	33	2/12	17	9/28	32	11/27	41	6/10	60	1/1	100

a - # Positive / Total in Age Group for each age group

TABLE F.

Frequency of Pulmonary and Non-Pulmonary Lesions in Mice of Various Age Groups

Mice with Pulmonary Lesions

Family	Age (Months) <10		10-12		13-15		16-18		19-21		22-24		25-27		28 & >		Total Mice
	No.[a]	%[b]	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
G	2	5	1	2	0	-	5	12	11	27	12	29	9	22	1	2	41
L	1	4	1	4	0	-	4	17	8	33	5	21	5	21	0	-	24
2	0	-	1	3	2	6	4	13	7	23	12	39	5	16	0	-	31
1	1	4	1	4	0	-	3	13	8	35	7	30	3	13	0	-	23
Total	4	3	4	3	2	2	16	13	34	29	36	30	22	18	1	1	119

Mice with Lesions of Other Systems

G	1	2	1	2	0	-	2	4	23	41	23	41	6	11	0	-	56
L	4	9	0	-	0	-	4	9	16	36	15	33	5	11	1	2	45
2	0	-	1	3	3	9	7	21	10	29	8	24	5	15	0	-	34
1	1	2	1	2	0	-	4	9	15	33	15	33	9	20	0	-	45
Total	6	3	3	2	3	2	17	9	64	36	61	34	25	14	1	1	180

a - Number of mice observed

b - Percent of total mice observed in each family

TABLE G.

Frequency of Pathologic Lesions in Mice of High and Low Incidence

Pulmonary Emphysema Families

Family	Not Rated & Neg.		PULMONARY LESIONS: Lung Tumors		Pneu.		Re Neoplasm (Lung)		Total Pulmonary		NON-PULMONARY LESIONS: Re Neo Other Organs		Leukemia		Misc. Lesions[c]		Total Non-Pulmonary		Total Mice
	No.[a]	%[b]	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
G	13	12	25	23	9	8	7	6	41	38	23	21	0	-	32	29	55	50	109
L	27	28	12	13	8	8	4	4	24	25	13	14	2	2	29	31	44	46	95
2	19	23	19	23	8	10	4	5	31	37	14	17	4	5	16	19	34	40	84
1	25	28	13	15	4	4	3	3	20	22	19	21	6	7	19	21	44	49	89

a - Number of mice observed

b - Percent of total mice observed in each family

c - Miscellaneous lesions are listed on the following page

footnote c, Table G - Definition of "miscellaneous lesions"

I. Neoplasms:
   A. - Adrenal
      1. Adrenal cortical adenomas
      2. Adrenal cortical carcinomas
      3. Pheochromocytomas
      4. Accessory adrenal gland

   B. - Ovary-uterus
      1. Cystic adenomatoid hyperplasia
      2. Hemangioendothelioma & hemangioma, uterus & oviduct
      3. Fibrosarcoma, uterus
      4. Leiomyosarcoma, uterus
      5. Adenocarc., ovary or uterus
      6. Theca granulosa cell tumor, ovary or uterus
      7. Fibroma, cervix

   C. - Testis
      1. Interstitial cell tumor

   D. - Digestive system
      1. Salivary glands
         a. myoepithelioma
      2. Liver
         a. hemangioendothelioma

   E. - Skin, subcutaneous and mucous
      1. Carcinomas
         a. vagina
         b. mammary glands
         c. ear canal
      2. Hemangioendothlelioma of subcutis

   F. - Reticuloendothelial system
      1. Spleen
         a. hemangioendotheliomas

II. A. Abcesses
   B. Generalized infections
   C. Granulomas
   D. Cardiac valvular stenosis

III. Degenerative
   A. Liver necrosis
   B. Nodular hyperplasia of liver

IV. Physiological alterations
   A. Hyperplasia
      a. erythroid of spleen and lymph nodes
   B. Siderosis
      a. lung

1003536140

TABLE H.

Frequency of Severe (3+ and 4+) Emphysema and Other Pathologic Lesions, By Age Group, in Families with High or Low Incidence of Pulmonary Emphysema

Characteristic	Incidence	AGE (Months) ≤10 Freq.[c]	%	10-12 Freq.	%	13-15 Freq.	%	16-18 Freq	%	19-21 Freq.	%	22-24 Freq.	%	25-27 Freq.	%	≥28 Freq.	%	TOTAL Freq.	%
Mice with Severe (3+ & 4+) Emphysema	High[a]	3/12	25	1/4	25	6/9	67	11/32	34	48/94	51	54/78	69	25/36	69	3/5	60	151/270	56
	Low[b]	0/3	-	1/3	33	0/3	-	2/12	17	9/28	32	11/27	41	6/10	60	1/1	100	32/87	37
Mice with Pulmonary Lesions	High	3/12	25	3/4	75	2/9	22	13/32	41	26/94	28	29/78	37	19/36	53	1/5	20	96/270	36
	Low	1/3	33	1/3	33	0/3	-	3/12	25	8/28	29	7/27	26	3/10	30	0/1	-	23/87	26
Mice with Non-Pulmonary Lesions	High	5/12	42	2/4	50	3/9	33	13/32	41	49/94	52	46/78	59	16/36	44	1/5	20	135/270	50
	Low	1/3	33	1/3	33	0/3	-	4/12	33	15/28	54	15/27	56	9/10	90	0/1	-	45/87	52
Mice c̄ Severe Emphysema and Concurrent Pulm. Lesions	High	2/12	17	1/4	25	1/9	11	7/32	22	18/94	19	24/78	31	14/36	39	1/5	20	68/270	25
	Low	0/3	-	1/3	33	0/3	-	1/12	8	2/28	7	3/27	11	3/10	30	0/1	-	10/87	12

a - Total of High Incidence Families G, L and 2
b - Family 1 (Low Incidence)
c - $\left(\frac{\text{\# with lesion}}{\text{Total observed}}\right)$ at each age group

TABLE I.

Comparison of Pathologic Findings by Family, at Various Ages

Pathology	AGE (Months)																
	< 10		10-12		13-15		16-18		19-21		22-24		25-27		28&>		Total Mice
	#[a]	%[b]	#	%	#	%	#	%	#	%	#	%	#	%	#	%	
FAMILY G																	
3+ or 4+ Emphysema	1	2	0	-	1	2	2	3	19	33	22	38	11	19	2	3	58
Pulmonary Lesions	2	5	1	2	0	-	5	12	11	27	12	29	9	22	1	2	41
Non-Pulmonary Lesions	1	2	1	2	0	-	2	4	23	41	23	41	6	11	0	-	56
FAMILY L																	
3+ or 4+ Emphysema	2	4	0	-	1	2	4	9	18	39	15	33	5	11	1	2	46
Pulmonary Lesions	1	4	1	4	0	-	4	17	8	33	5	21	5	21	0	-	24
Non-Pulmonary Lesions	4	9	0	-	0	-	4	9	16	36	15	33	5	11	1	2	45
FAMILY 2																	
3+ or 4+ Emphysema	0	-	1	2	4	9	5	11	11	23	17	26	9	11	0	-	47
Pulmonary Lesions	0	-	1	3	2	6	4	13	7	23	12	39	5	16	0	-	31
Non-Pulmonary Lesions	0	-	1	3	3	9	7	21	10	29	8	24	5	15	0	-	34
FAMILY 1																	
3+ or 4+ Emphysema	1	3	1	3	1	3	2	6	9	28	11	34	6	19	1	3	32
Pulmonary Lesions	1	4	1	4	0	-	3	13	8	35	7	30	3	13	0	-	23
Non-Pulmonary Lesions	1	2	1	2	0	-	4	9	15	33	15	33	9	20	0	-	45
TOTAL OF 4 FAMILIES																	
3+ or 4+ Emphysema	4	2	2	1	7	4	13	7	57	31	65	36	31	17	4	2	183
Pulmonary Lesions	4	3	4	3	2	2	16	13	34	29	36	30	22	18	1	1	119
Non-Pulmonary Lesions	6	3	3	2	3	2	17	9	64	36	61	34	25	14	1	1	180

a - Number of mice observed in each age group

b - Percent of total mice in each category

1003536142

TABLE J. Concurrent Findings of Emphysema with Other Pathology of the Pulmonary System.

	Family G			Family L			Family 2			Family 1		
	Severity		Total	Severity		Total	Severity		Total	Severity		Total
	Neg. to 2+	3+ & 4+		Neg. to 2+	3+ & 4+		Neg. to 2+	3+ & 4+		Neg. to 2+	3+ & 4+	
Alveolar Adenoma	9	8	17	2	6	8	1	7	8	6	4	10
Alveolar Adenocarcin.	1	7	8	1	3	4	1	10	11	0	3	3
Pneumonia	4	5	9	4	4	8	0	8	8	2	2	4
R.E. Neo.; Lung	0	7	7	1	3	4	1	3	4	1	2	3
All lung Lesions	14	27	41	8	16	24	3	28	31	9	11	20
All Tumors	10	22	32	4	12	16	3	20	23	7	9	16
Animals with Multiple Lesions			1			1			1			1

1003536143

Text - Figure 1

Photomicrographs illustrating typical pathologic findings associated with each classification of pulmonary emphysema.

A - Negative - essentially normal - note intact alveolar walls. A slight degree of atelectasis is present.

B - 1+ - minimal overdistention of alveolar walls is present.

C - 2+ - slight overdistention of alveolar walls is present.

D - 3+ - mild to moderate overdistention of alveolar walls is seen. Also noted are focal areas of atelectasis.

E - 4+ - areas of severe overdistention of alveolar walls is present. Some atelectasis is noted.

F - 4+ - (Bullous emphysema) prominent areas of overdistention are seen beneath the pleura.

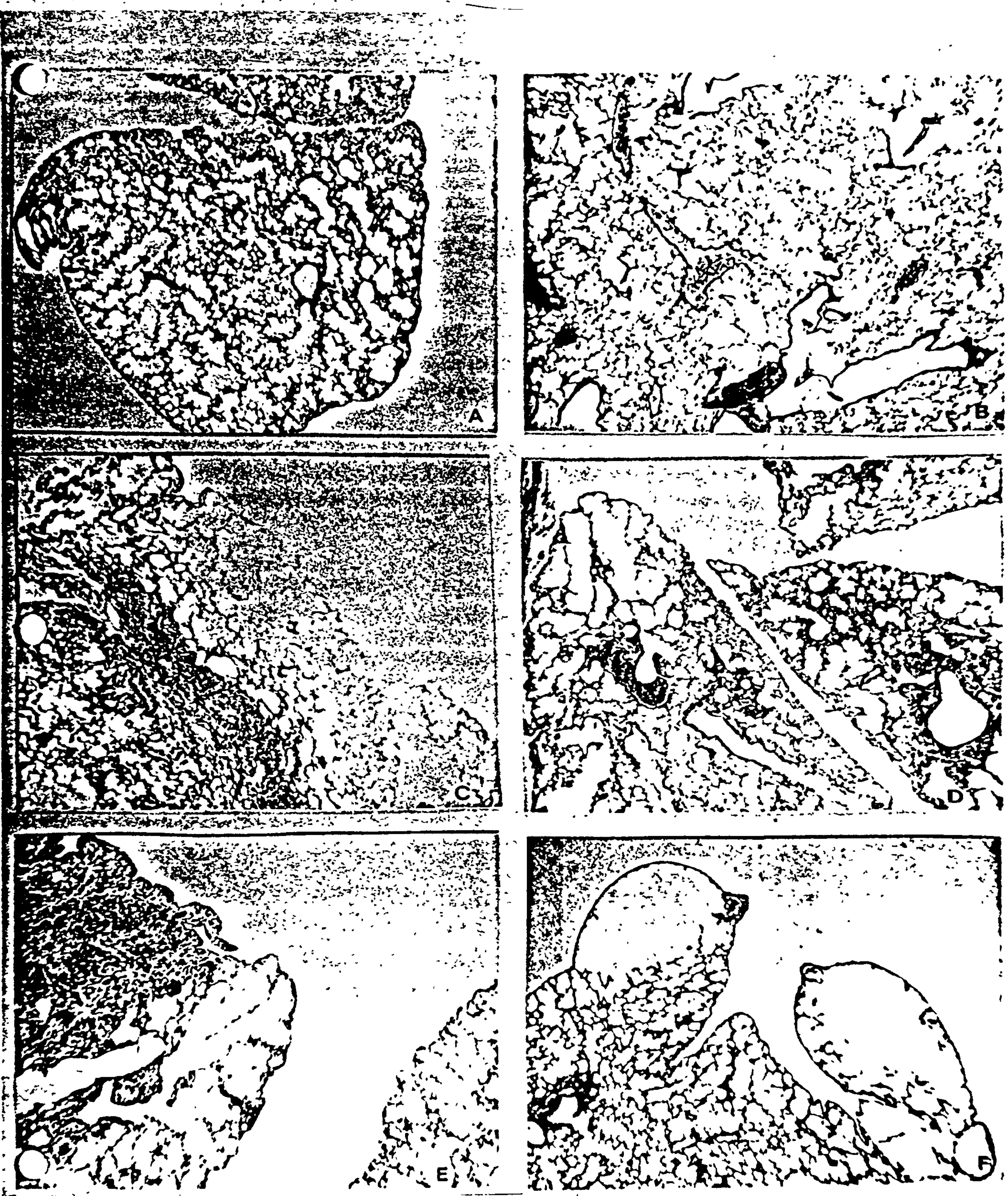


FIGURE 1

1003536145

Text - Figure 2

Comparative incidence and severity of emphysema, High (Families G, L and 2) and Low (Family 1) Incidence Families.

The bars for High Incidence Families indicates the range of observations between the 3 Families. Each point is the $\left(\frac{\text{No. with emphysema}}{\text{Total population observed}}\right)$. The total number of mice in the High incidence families is 258; Family 1 is 84.

1003536146

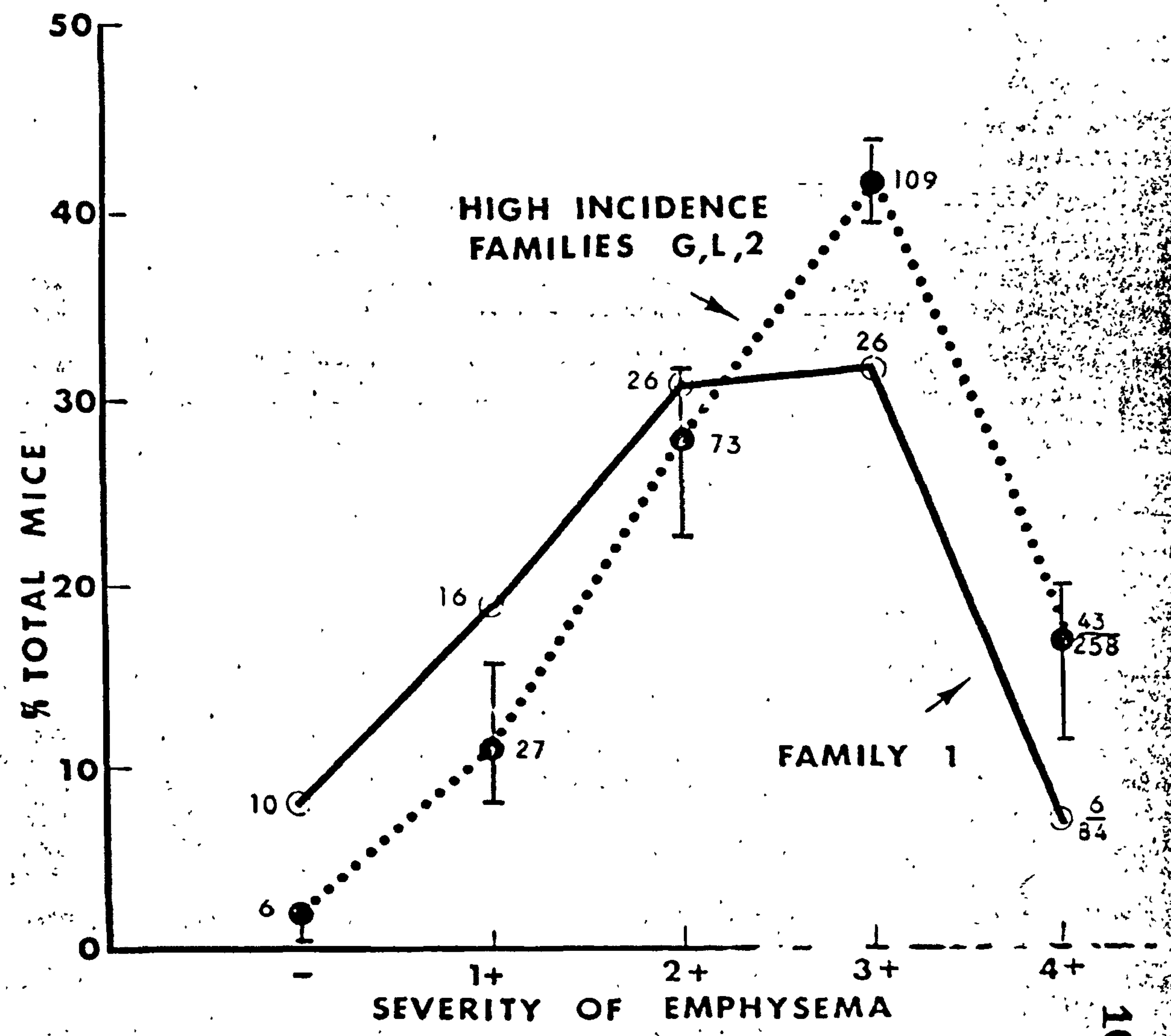


FIGURE 2

1003536147

Text - Figure 3

Mean age of mice with each degree of emphysema in High (Families G, L and 2) and Low (Family 1) Incidence Families.

The bars for High Incidence Families indicates the range of observations between the 3 Families. Numbers beside the points indicate the number of mice comprising the group.

1003536148

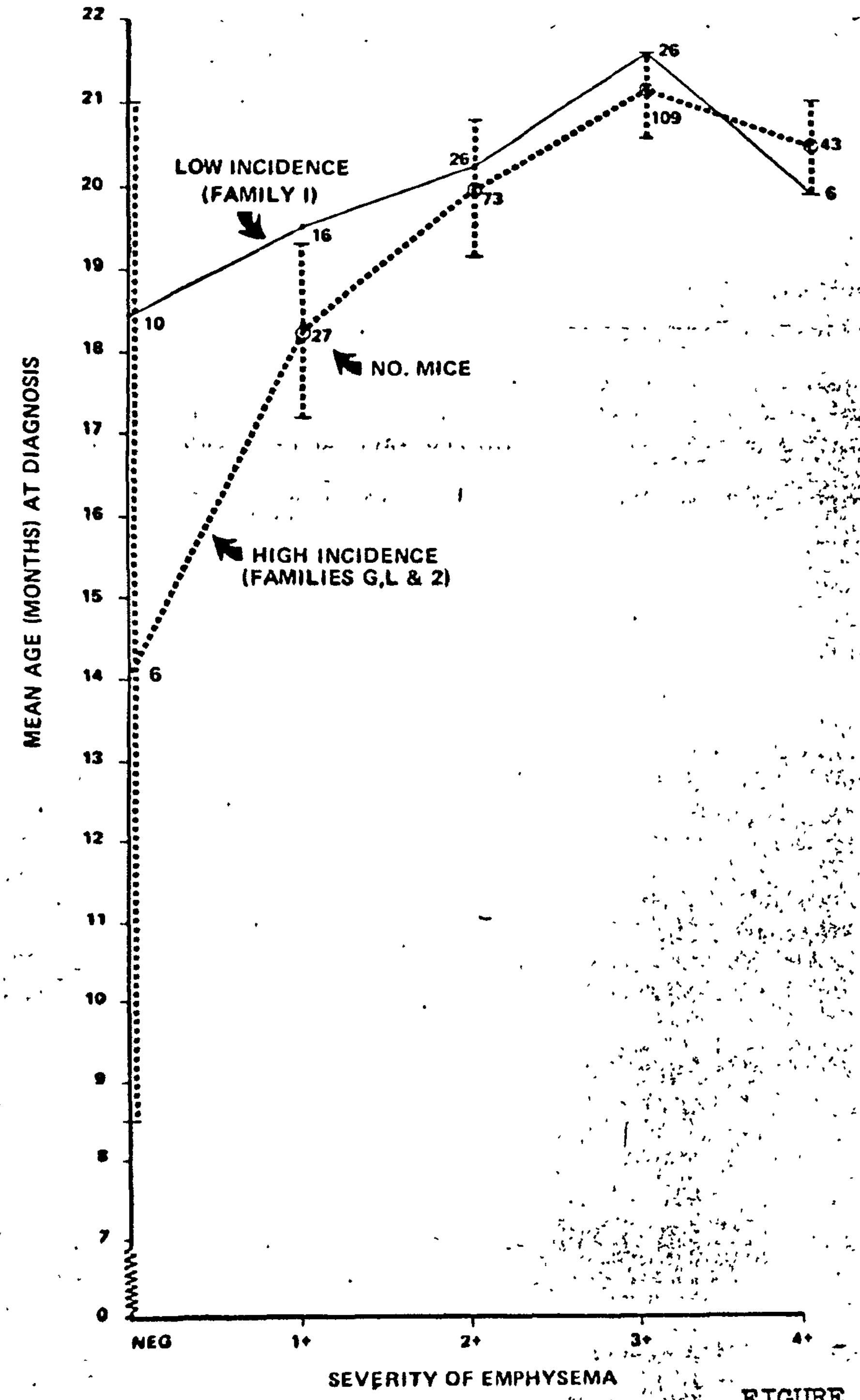


FIGURE 3

1003536149

Text - Figure 4

Comparative Incidence and Severity of Emphysema between male and female mice.

The bars show the percent of total male or female mice with each severity of emphysema. The numbers at the base of each bar are the number of mice comprising the group.

1003536150

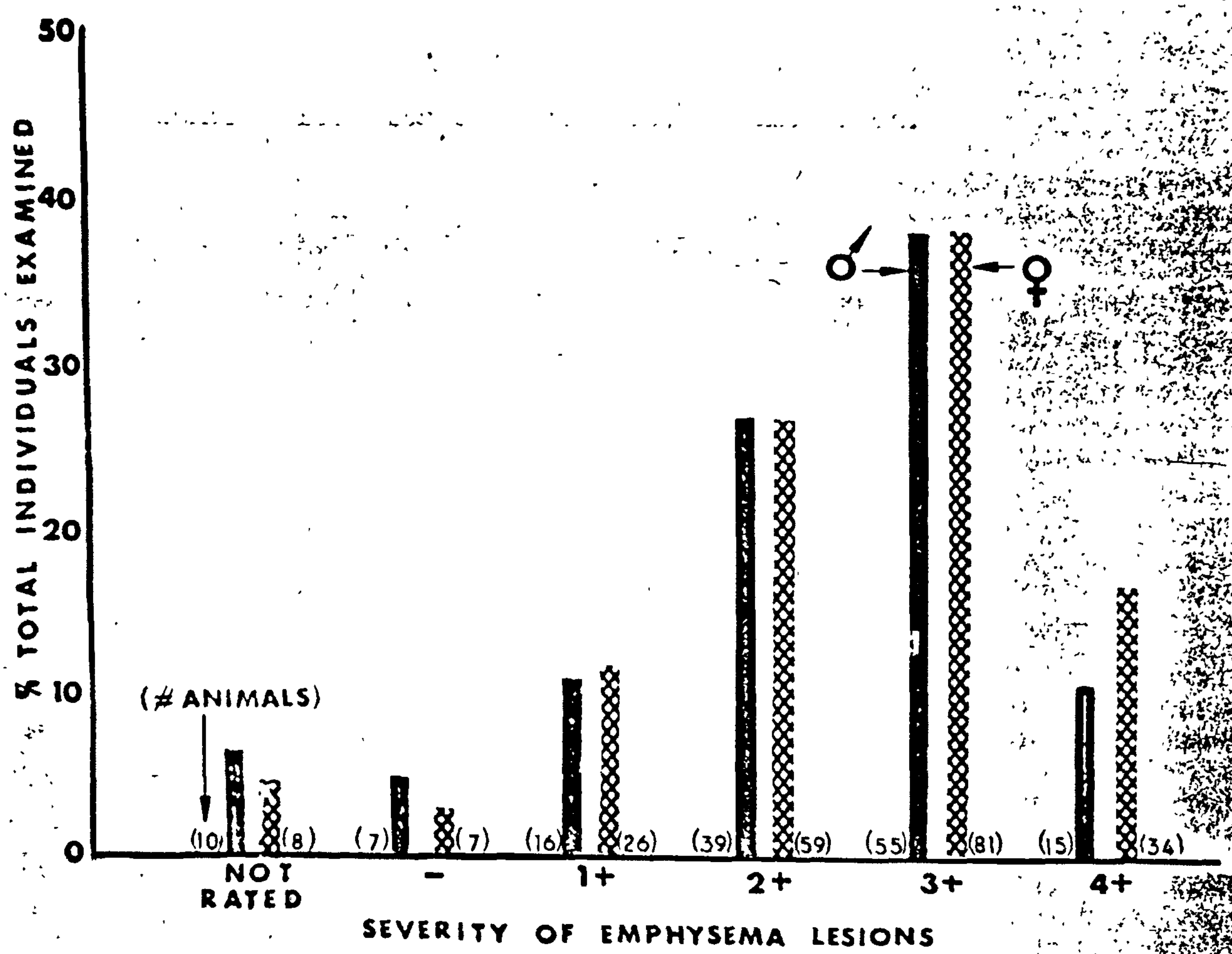


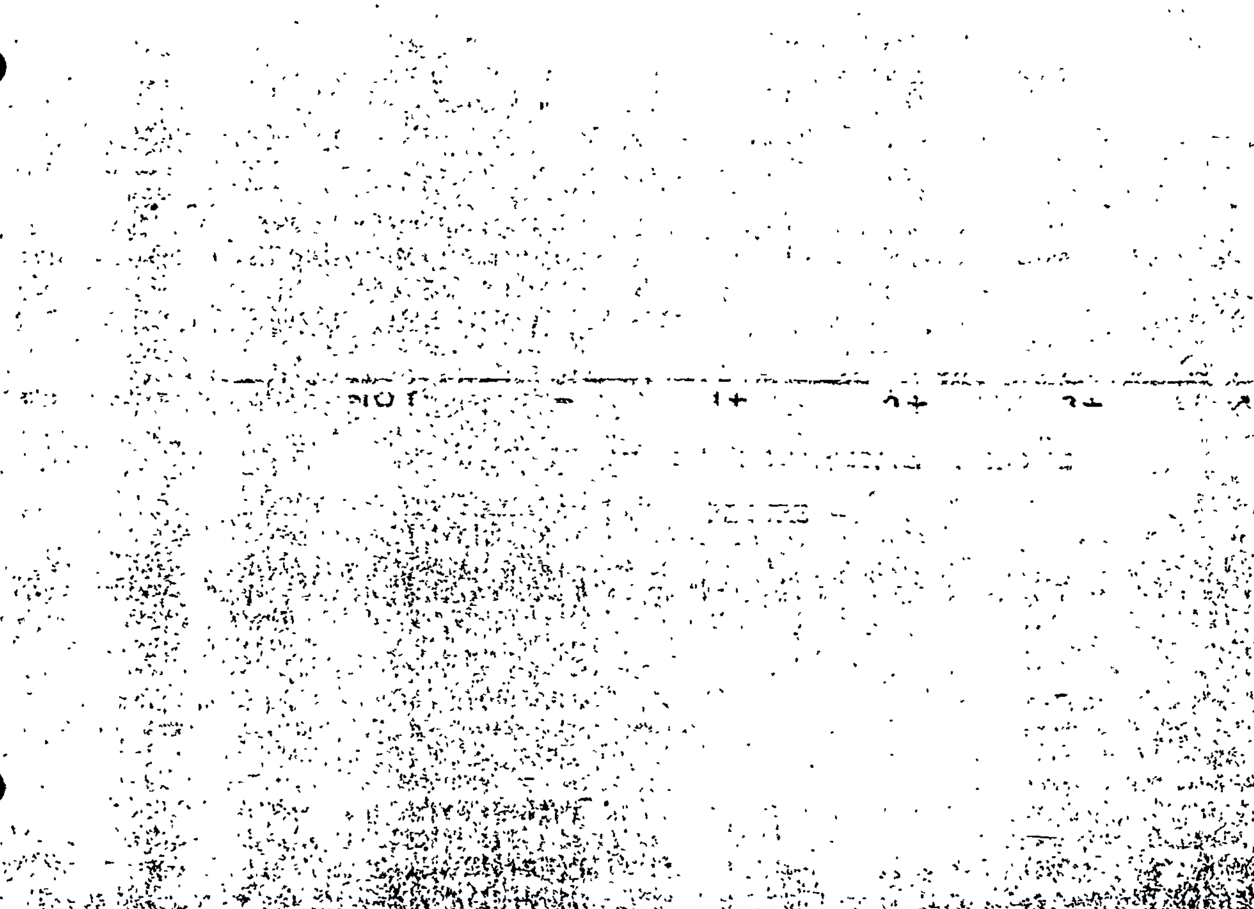
FIGURE 4

1003536151

Text - Figure 5

Comparative Incidence and Severity of Emphysema in mice between formalin-fixed inflated or uninflated lung sections.

The bars show the percent of total inflated or uninflated mouse lung specimens examined with each severity of emphysema. The numbers at the base of each bar are the numbers of specimens comprising the group. There was a total of 92 inflated specimens; 268 uninflated.



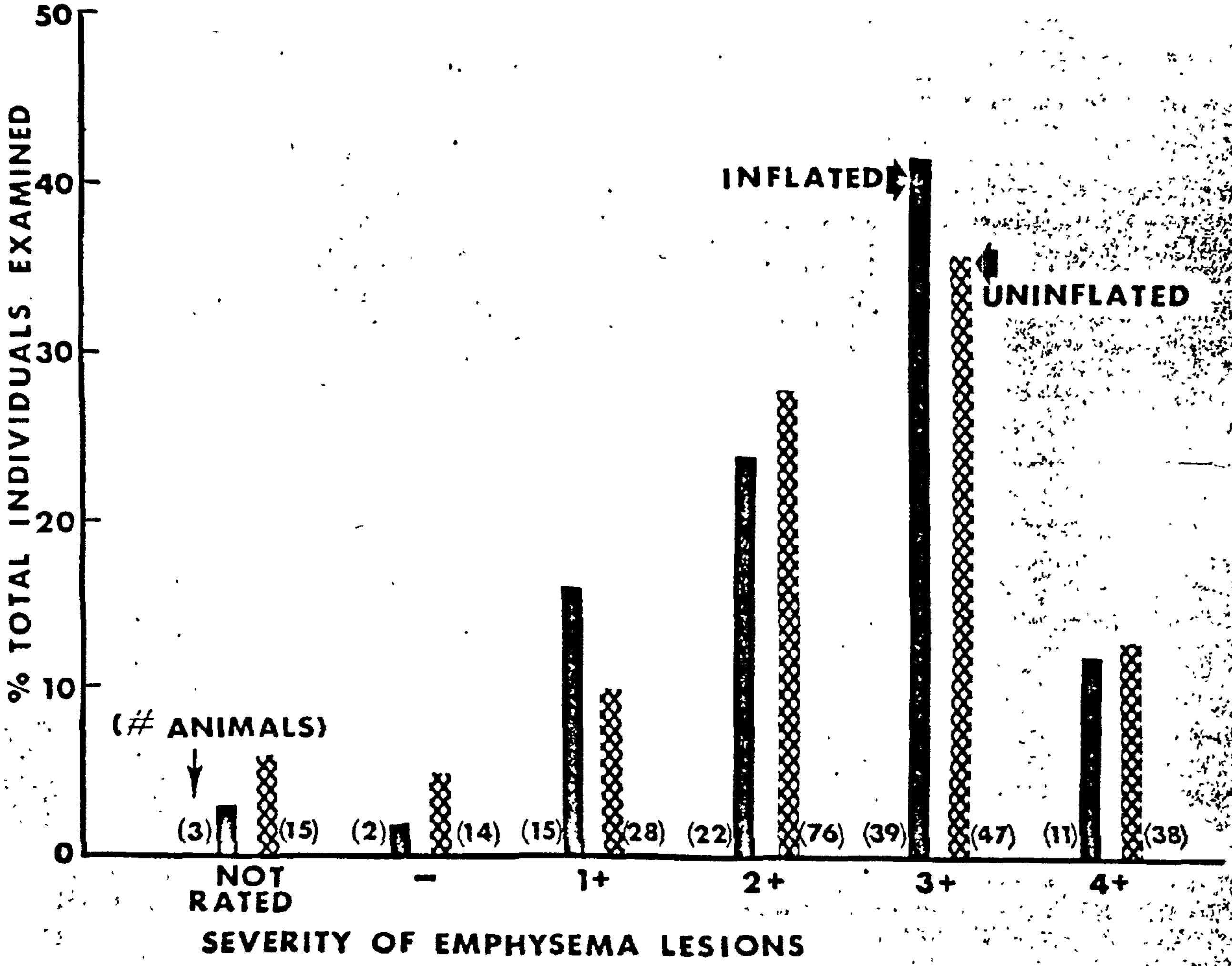


FIGURE 5

1003536153

Text - Figure 6

Comparative Incidence of Severe (3+ or 4+) Pulmonary Emphysema, by age group, between High (Families G, L, and 2) and Low (Family 1) Incidence Families.

The number at each point is the $\left(\frac{\text{No. with severe emphysema}}{\text{Total observed}}\right)$ for each age group. Each age group spans a time of 3 months. The bars for High Incidence Families indicates the range of observations between the 3 families.

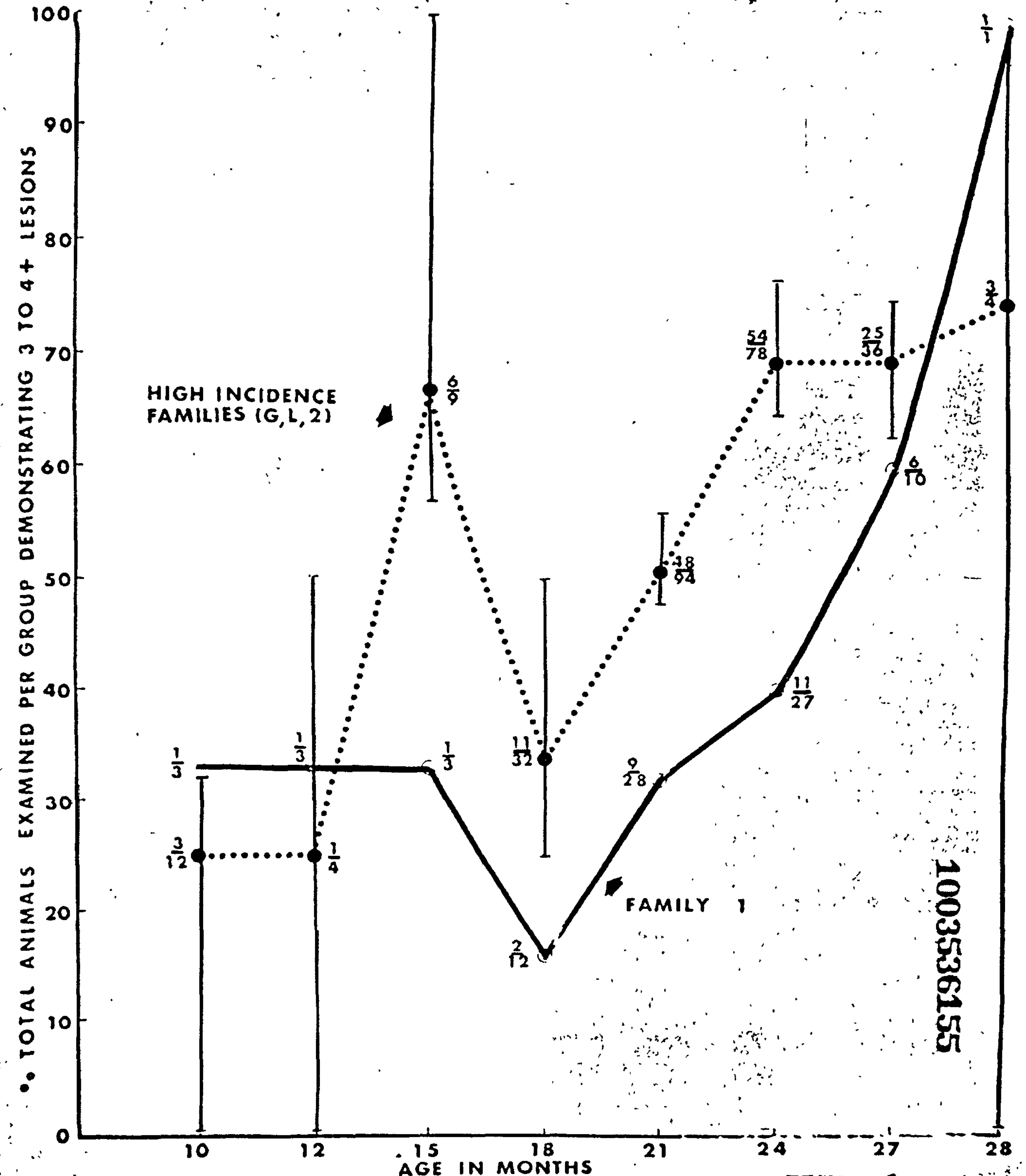


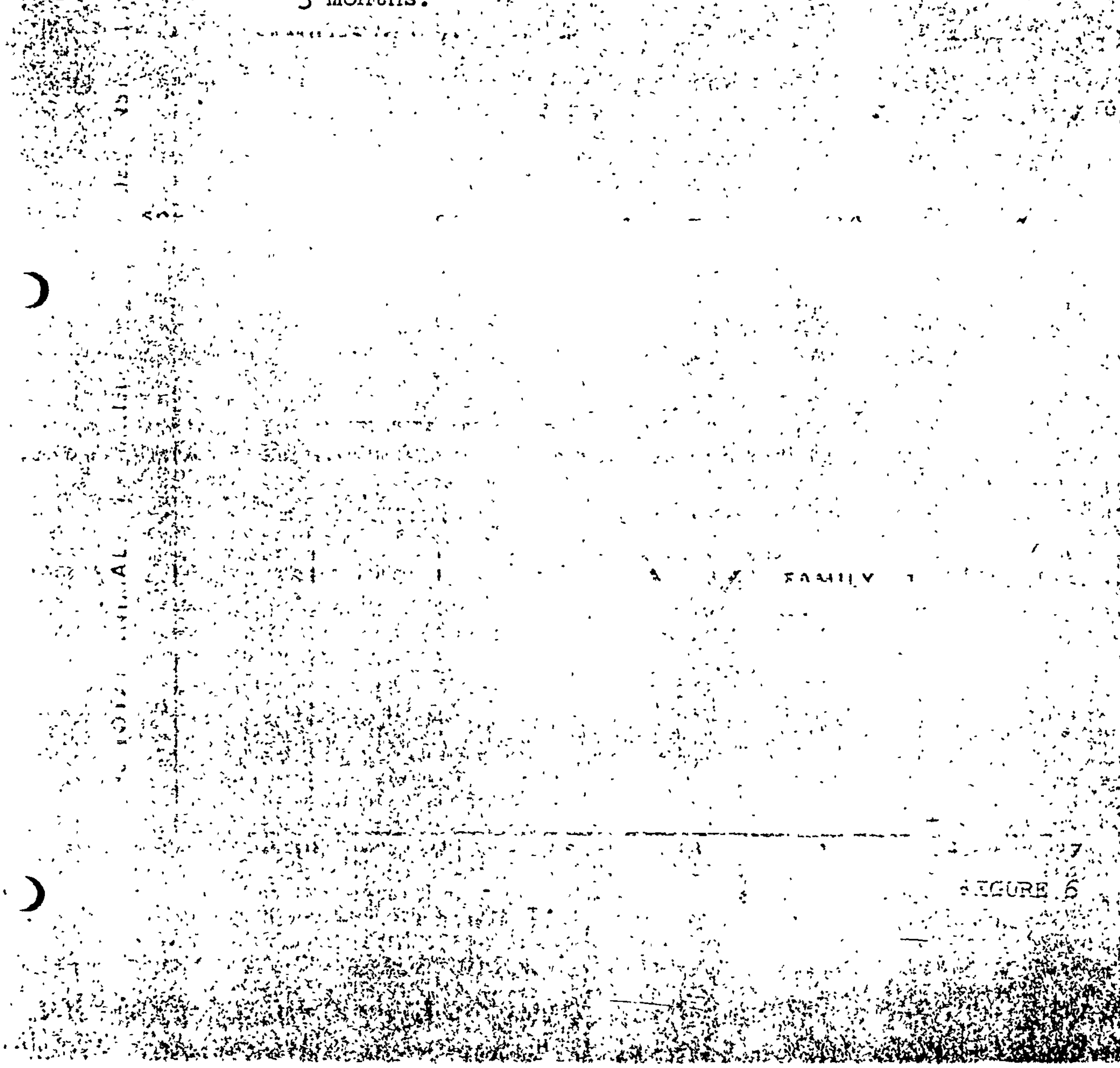
FIGURE 6

1003536155

Text - Figure 7

Comparative frequency of pulmonary lesions, by age group, in High (Families G, L and 2) and Low (Family 1) Incidence Families.

The number at each point is the $\left(\frac{\text{number positive}}{\text{total observed}}\right)$ for each age group. Each age group spans a time of 3 months.



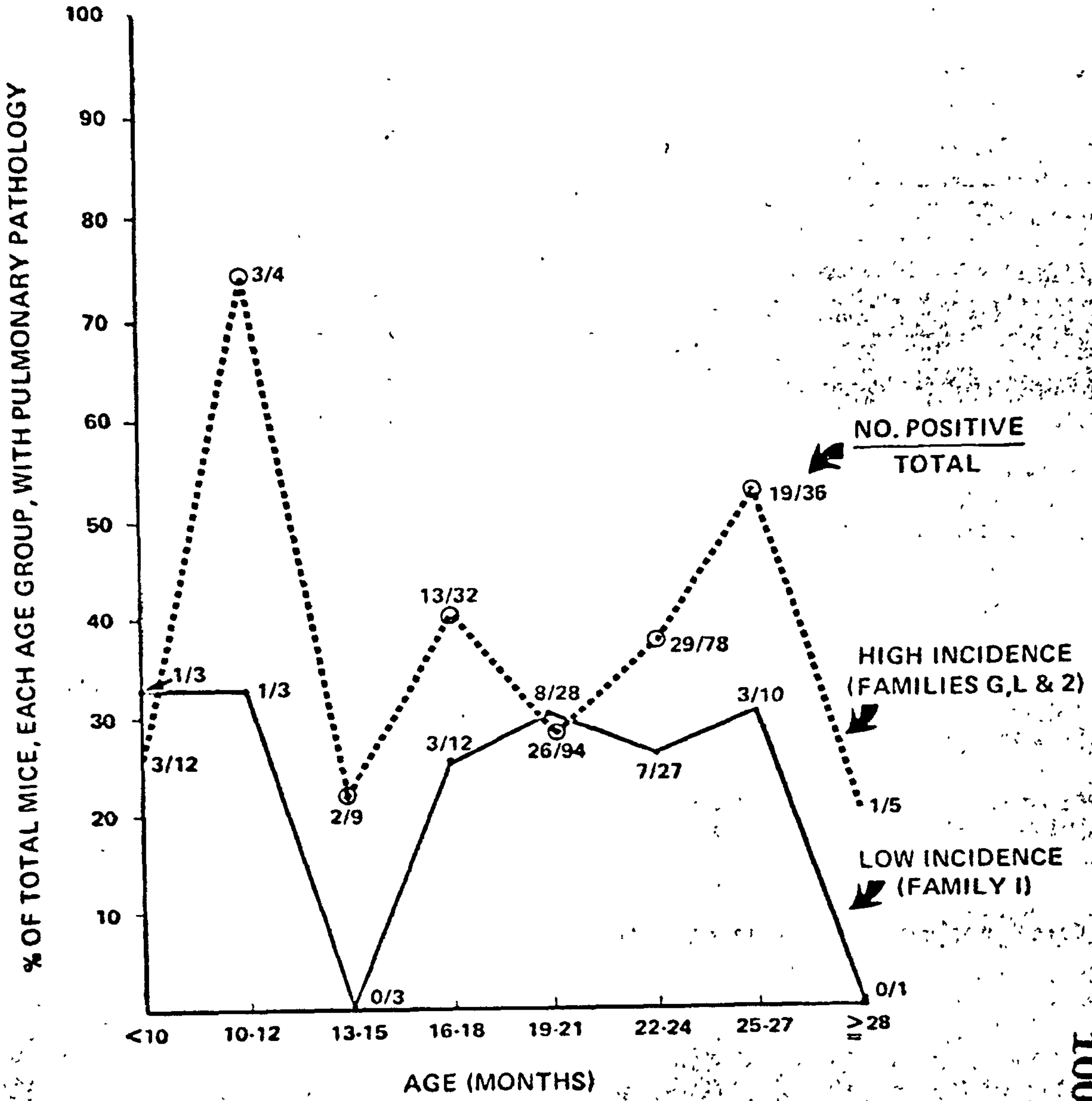


FIGURE 7

1003536157

Text - Figure 8

Comparative frequency of non-pulmonary lesions, by age group, in High (Families G, L and 2) and Low (Family 1) Incidence Families.

The number at each point is the $\left(\frac{\text{number positive}}{\text{total observed}}\right)$ for each age group. Each age group spans a time of 3 months.

1003536158

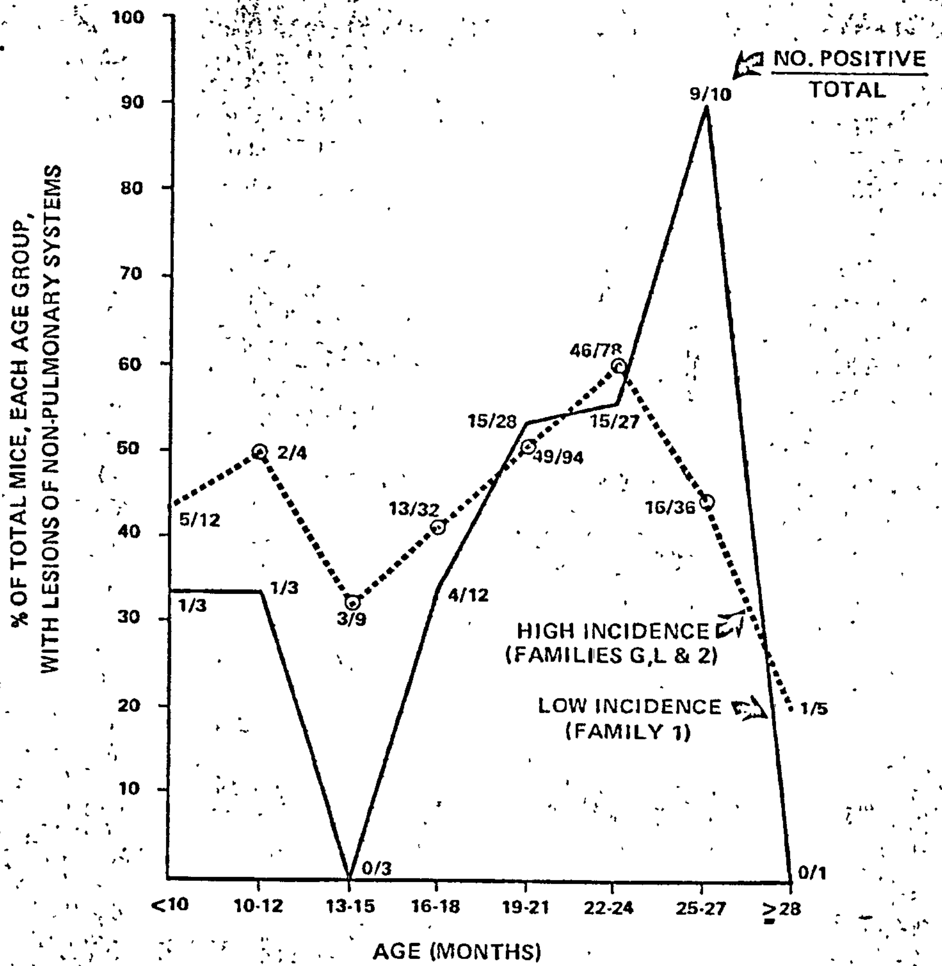


FIGURE 8

1003536159

Text - Figure 9

Comparative frequency of occurrance of different type pulmonary lesions, by age group, in the High (Families G, L and 2) and Low (Family 1) Pulmonary Emphysema Incidence Families.

The % frequency represents the $\left(\frac{\text{number with lesion}}{\text{total observed}}\right)$ for each family. The number at the base of each bar indicates number of mice comprising the group. The average of the High Incidence Families (G, L and 2) is shown as a separate bar. Multiple lesions were found in some of the mice.

1003536160

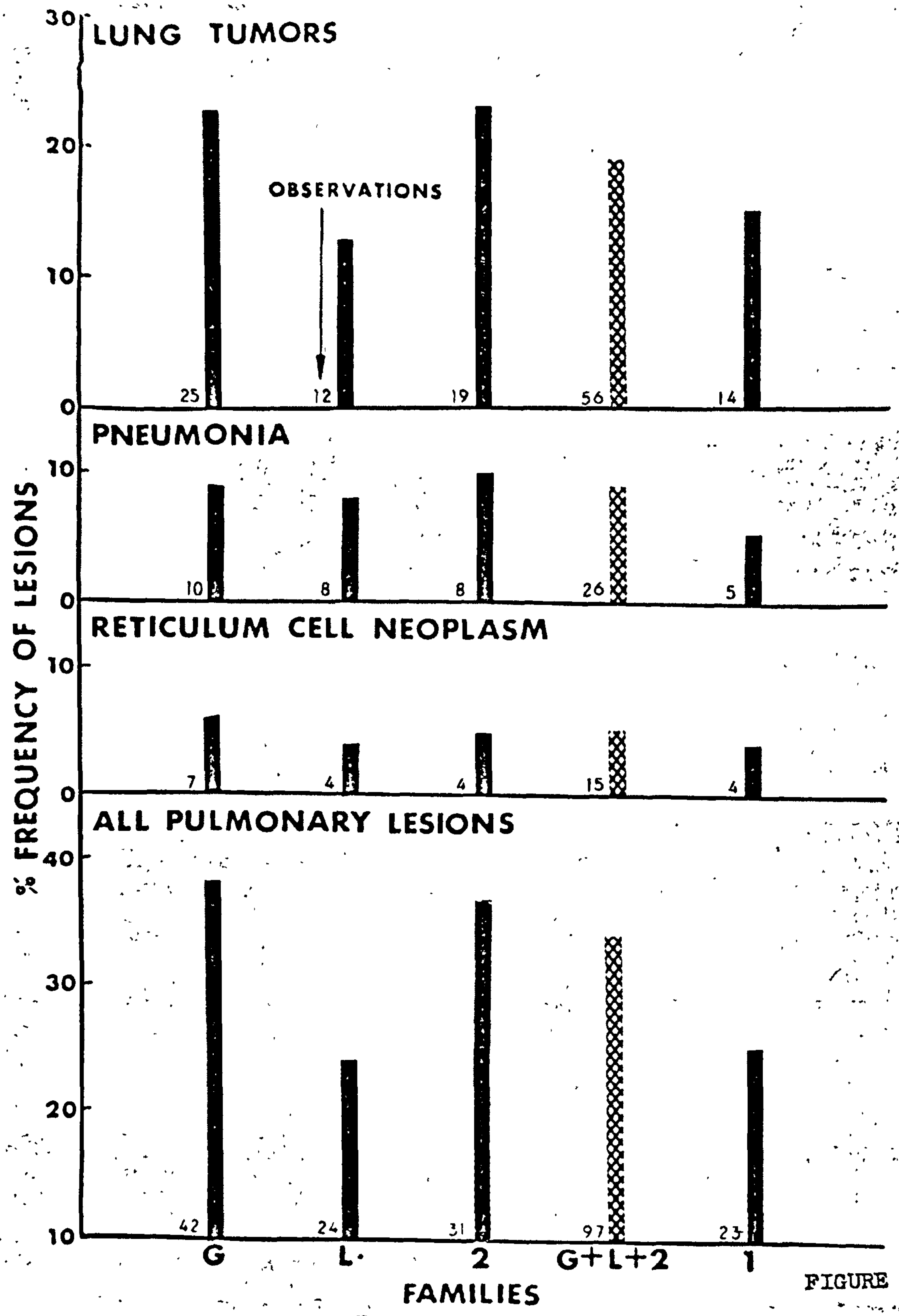


FIGURE 9

1003536161

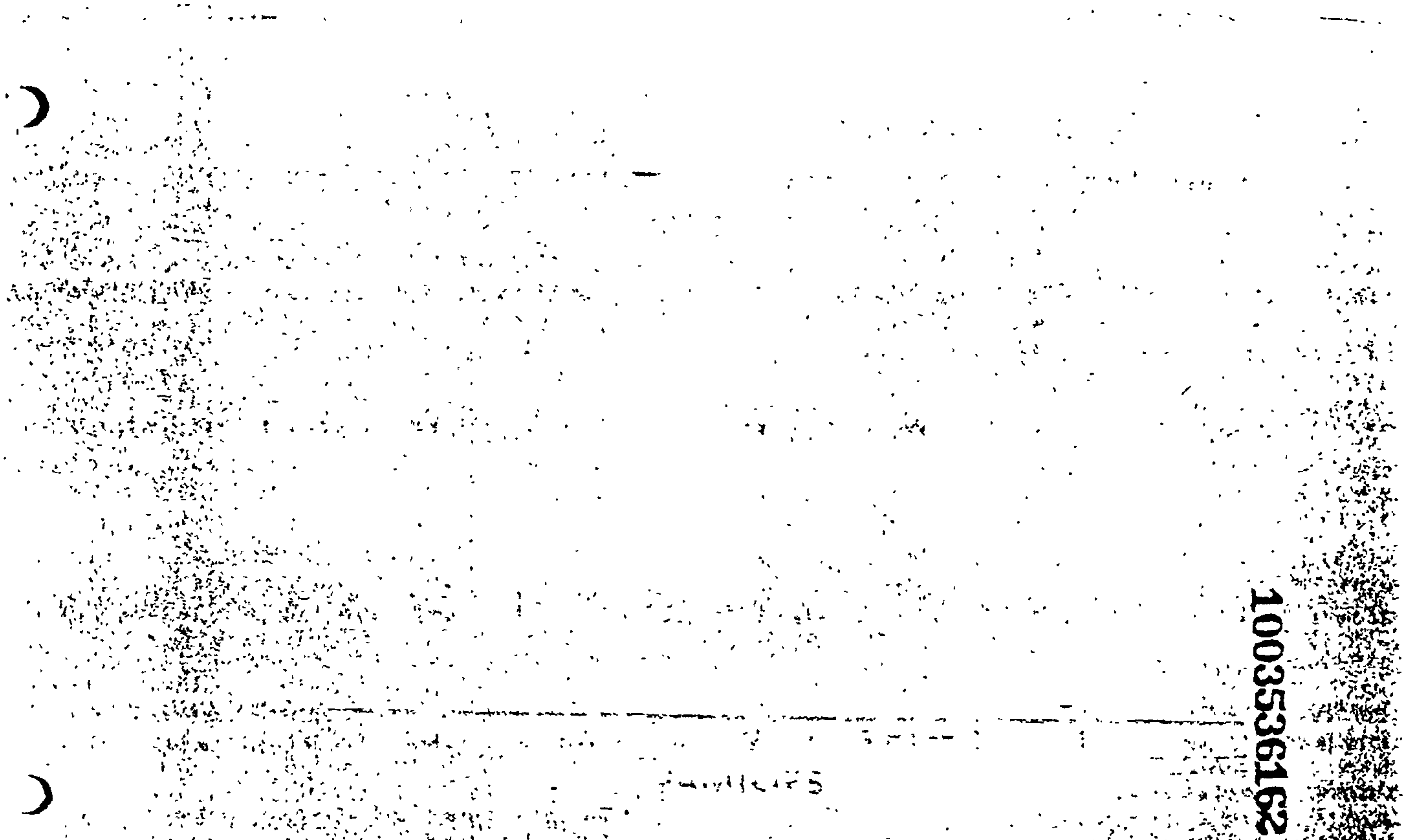
Text - Figure 10

Comparative frequency of occurrence of severe (3+ or 4+) emphysema with concurrent lung lesions, by age group, in High (Families G, L and 2) and Low Pulmonary Emphysema Incidence Families.

The number at each point is the

$$\left(\frac{\text{number with severe emphysema and pulmonary lesion(s)}}{\text{total observed}}\right)$$

for each age group. Each age group spans a time of 3 months.



1003536162

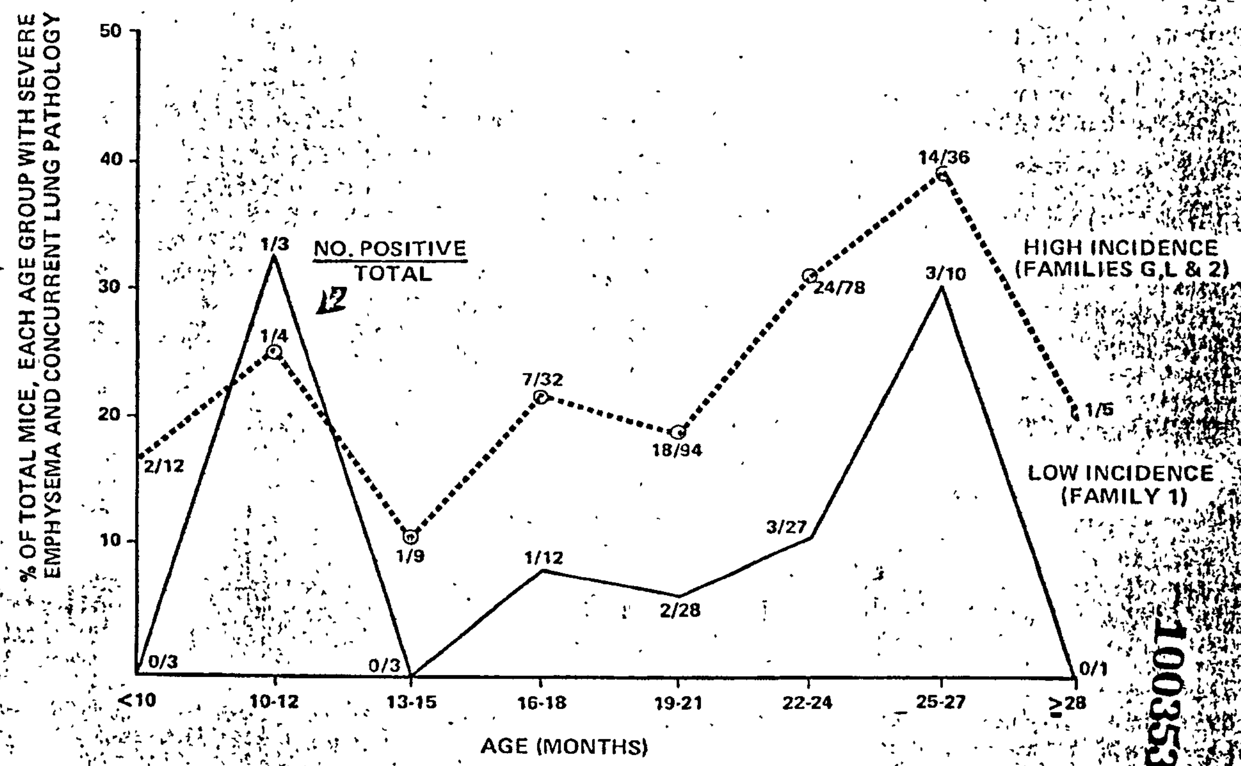


FIGURE 10

1003536163

Text - Figure 11

Comparative $alpha_1$ trypsin inhibition between male and female mice of the different families.

Each bar indicates the mean of all male or female mice in each group. The mean of the High Incidence Families (G, L and 2) is shown in a separate bar.

1003536164

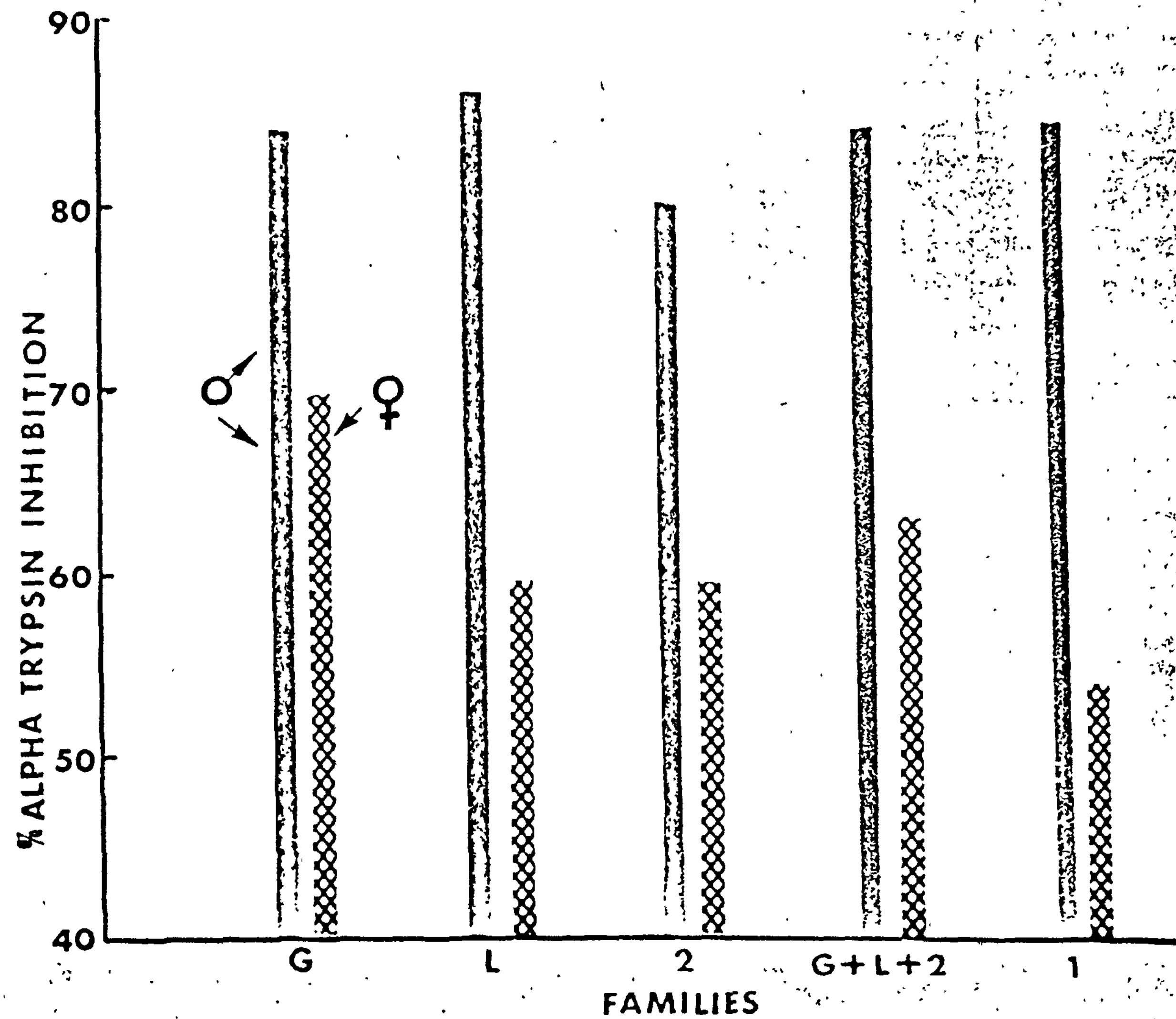


FIGURE 11

1003536165

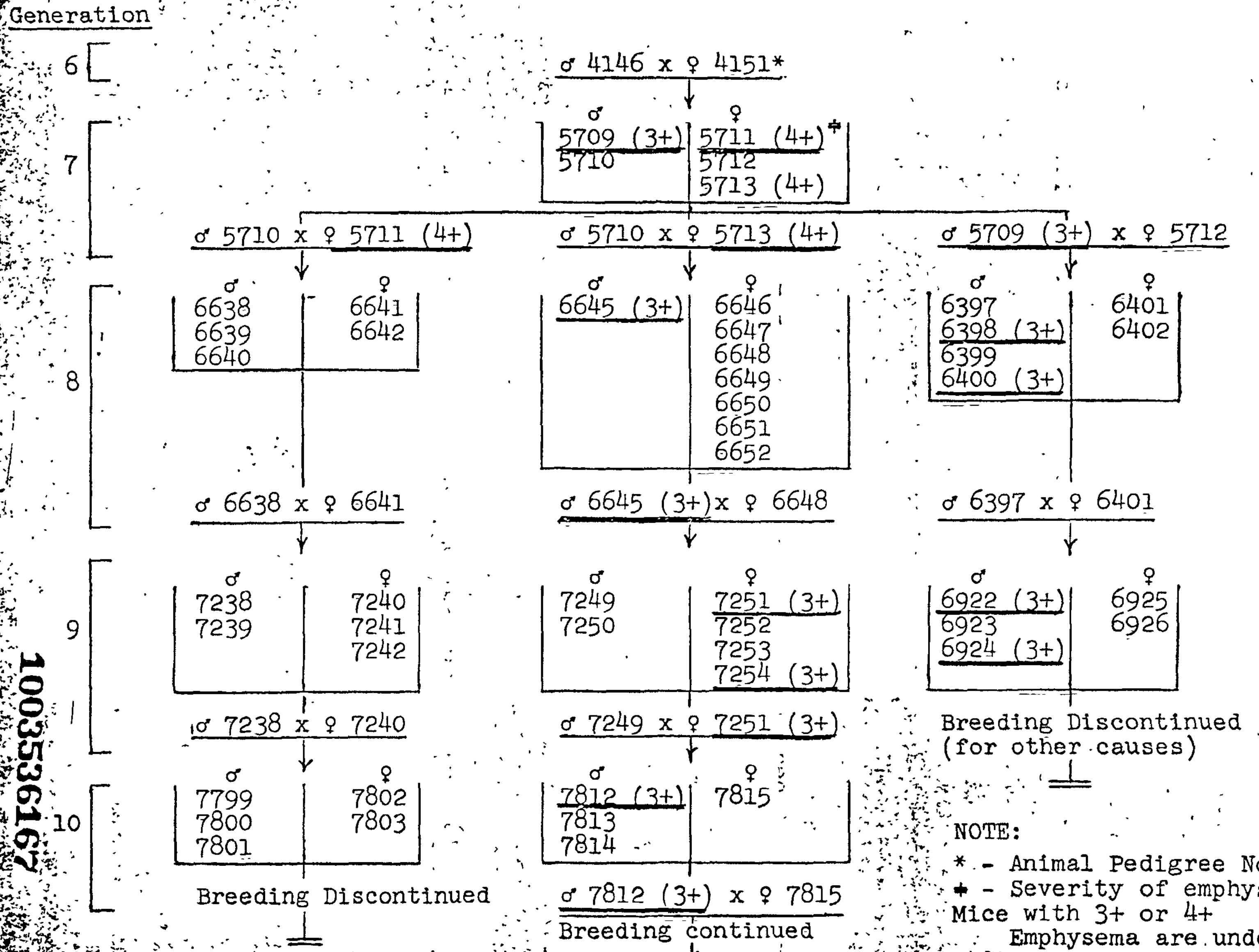
Text - Figure 12

Excerpt from the Pedigree chart of Family L, illustrating the segregation of a tendency toward severe emphysema within a family.

Underneath each mating, the individual mice comprising the progeny are shown by pedigree number and sex. Mice later diagnosed as having 3+ or 4+ emphysema are underlined. Note that in Generation 7, ♂ No. 5710 was bred to 2 of his sisters (♀ No. 5711 and ♀ No. 5713).

This figure illustrates the fact that since a diagnosis of severity of emphysema is established only after the mouse has died, usually well past breeding age, selection of breeder matings must be based on retrospective data of their ancestors, 3 to 5 generations past. The figure illustrates that even this information is of great value in attempting to accentuate the trend toward a high or low incidence of pulmonary emphysema. Physiological or biochemcial measurements which prove to be predictive of probable future emphysema during the active breeding age of the mice would be of immense value in such genetic studies.

FIGURE 12 - Excerpt from Pedigree chart of Family L



NOTE:
* - Animal Pedigree No.
✚ - Severity of emphysema
Mice with 3+ or 4+ Emphysema are underlined.

MICROBIOLOGICAL ASSOCIATES
Division of DYNASCIENCES Corporation
4733 Bethesda Avenue / Bethesda, Maryland 20014 / (301) 654-3400

September 13, 1974

To: Dr. John Kreisher
Associate Scientific Director
Council for Tobacco Research U.S.A., Inc.
110 East 59th Street
New York, New York 10022

From: Dr. Bernard Sass

Subject: Consultation with Dr. Ralph Powell -- National Institutes of Health

Dr. Powell and I reviewed some lung sections from aged BALB/c mice suspected of having emphysema. We also discussed methods of fixation and perfusion of lungs. Whole human lung sections some of which demonstrated emphysema were viewed. Perfusion of human lungs with gluteraldehyde in a tank with a continuous flow constant pressure pump driven system was also demonstrated.

After examining some of the slides the following points were made:

1. Currently, emphysema is defined as both overdistention of alveoli and destruction of their walls.

2. Care must be taken not to misinterpret discontinuity due to small airways (i.e. alveolar ducts) as emphysema.

3. Emphysema was believed present in some of the slides examined.

4. Dr. Powell believed this project had merit, but more refinements were needed. These included:

   1. Maintenance of constant pressure when inflating the lungs.

   2. Further consultations to evaluate the degree of emphysema present.

BS:pop

cc: Dr. R.M. Nims
Dr. R. Powell, NIH
Dr. H. L. Stewart, NCI
Dr. C. Whitmire (Bethesda)

1003536168

MICROBIOLOGICAL ASSOCIATES • INC
Subsidiary of DYNASCIENCES Corporation
4733 Bethesda Avenue / Bethesda, Maryland 20014 / (301) 654-3400

September 17, 1974

Dr. John Kreisher
Council for Tobacco Research U.S.A., Inc.
Associate Scientific Director
110 East 59th Street
New York, New York 10022

Dear John:

This is in response to your inquiry about the incidence of Sendai infection in the pedigreed BALB/c mice.

These mice are sampled twice yearly. For at least the last 5 years, there has been no serologic evidence of Sendai injection. The pneumonia, therefore, is not related to Sendai. Its origin is either bacterial or one of the murine viruses other than Sendai.

Sincerely,

Bob

Robert M. Nims, D.V.M.
Director, Walkersville Fac.
Walkersville, Md. 21793

RMN/bdh

1003536169

1003536170

HUMAN AHH
STUDIES

1003536171

PIKE - U.S.C.

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
SEP 17 1974
RECEIVED

Application For Renewal of Research Grant
(Use extra pages as needed)

First Renewal [x] Second Renewal [ ] Date: 9/13/74

1. Principal Investigator (give title and degrees): M. C. Pike, Ph.D.
Professor, Community Medicine
and Pediatrics

2. Institution & address: University of Southern California
School of Medicine
2025 Zonal Avenue
Los Angeles, California 90033

3. Department(s) where research will be done or collaboration provided:
Department of Pathology

4. Short title of study: Study of relationship between susceptibility to certain cancers and aryl hydrocarbon hydroxylase (AHH) activity

5. Proposed renewal date: November 1, 1974

6. How results to date have changed earlier specific research aims: No change in basic epidemiological research aims which are attached.

7. How results to date have changed earlier working hypothesis: No change

1003536172

8. Any additional facilities now required? Describe briefly: None

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

The key personnel in this research are:
M. C. Pike, Ph.D.
J. C. Brown, M.D.
R. J. Gordon, Ph.D.

C.V.'s of each are appended.

10. Append outline of experimental protocol for ensuing year. See attached

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

None

12. Summary progress report (append in standard form as separate document, unless recently submitted).

1003536173

R: REDACTED MATERIAL

3.

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Professional		
M. C. Pike, Ph.D.	20	–
J. C. Brown, M.D.	20	–
R. J. Gordon, Ph.D.	10	–
Technical		
S. Brown, Tech I	100	$ R
R. Hanisch, R.N. Nurse Epidemiologist II	100	
To be recruited-Tech II	100	
Part-time laboratory assistant ~~xxxxx~~ $2.50/hour x 15 hours/week		1,950
	Subtotal	$33,269
Projected 6% salary increase for period July 1-October 31, 1975 (base $11,090)		665
	Sub-Total for A	$33,934
B. Consumable supplies (by major categories)		
Supplies for shipments, boxes, wrappings, tubes and needles		$ 780
Glassware, chemicals, media, automatic pipettes and miscellaneous laboratory supplies		13,200
6% tax (base $13,980)		839
	Sub-Total for B	$14,819
C. Other expenses (itemize)		
Specimen shipments to Microbiological Associates - $15.75/shipment x 3 shipments x 52 weeks		$ 2,457
Travel: An estimated 10,000 miles @ 12¢/mile		1,200
	Sub-Total for C	$ 3,657
	Running Total of A + B + C	$52,410
D. Permanent equipment (itemize)		
International centrifuge:		
PR 6000		$ 3,091
C 2055 head		190
Shields (12 @ $5.00 ea)		60
Triunion (12 @ $6.75 ea)		81
6% tax (base $3,422)		205
	Sub-Total for D	$ 3,627
E. Indirect costs (15% of A + B + C)	E	7,862
	Total amount	$63,899

1003536174

R: REDACTED MATERIAL

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Comprehensive Field and Laboratory Research Program on the Etiology and Epidemiology of Human Cancer	N.C.I. Contract PH 43-NCI-68-1030	2,006,206	10/1/74-6/30/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Proposed Grant: USC Cancer Center Epidemiology and Biostatistics Unit	N.C.I.	2,405,636	1/1/75-12/31/77

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name M. C. Pike, Ph.D.

Signature MCPike Date 9/13/74

Telephone R R R
Area Code Number Extension

Checks payable to
University of Southern California
School of Medicine

Mailing address for check:
2025 Zonal Avenue
Los Angeles, California 90033
Attention: Gary King

Responsible officer of institution

Typed Name Zohrab A. Kaprielian

Title Vice President, Financial and Legal Affairs

Signature ______ Date ______

Telephone ______
Area Code Number Extension

1003536175

STUDY OF THE RELATIONSHIP BETWEEN
SUSCEPTIBILITY TO CERTAIN CANCERS AND
ARYL HYDROCARBON HYDROXYLASE (AHH) INDUCIBILITY

December 1973

1003536176

Background:

Evidence for AHH inducibility in human peripheral blood lymphocytes being closely associated with susceptibility to bronchogenic carcinoma has been recently provided by Kellerman, et al (New Engl. J. Med., November 1, 1973, Page 934).

These workers maintain that such AHH inducibility is controlled by a single genetic locus with 2 alleles; a low inducible allele A and a high inducible allele B. If the lung cancer risk is taken as 1 for AA persons, then it is estimated that the risk for an AB person is 16 and for a BB person is 36. These are very high risk values, at least as high as those associated with cigarette smoking.

Further studies of AHH inducibility are most certainly needed.

1-Lung cancer: Can the results of Kellerman and his colleagues be repeated? If they can, then what is the relationship between AHH inducibility and the risk to lung cancer as it is affected by 1) increasing age, 2) cigarette smoking habits, 3) cell type, and 4) exposure to occupational or atmospheric carcinogens.

2-Other "chemically induced tumors": Is there any relationship between AHH inducibility in peripheral blood lymphocytes and tumors of the larynx, bladder, esophagus, nasopharynx, colon, etcetera?

Proposed Study:

Our initial task is to establish a repeatable test of AHH inducibility in human peripheral lymphocytes. To this end collaborative arrangements have been made with Dr. Richard E. Kouri at Microbiological Associates. Once the test is established we propose to obtain specimens of 15-30 ml of heparinized blood from two hospitalized population groups.

1-Patients with lung cancer and controls, and,

2-Persons with cancer of other sites such as those listed above.

Each patient will be interviewed to obtain basic information on age, race, socio-economic status, residence, occupation, and smoking history prior to the obtaining of a blood sample. All patients will be asked to sign a consent form after the details of the study have been explained to them. All information collected will be kept strictly confidential although the results of the assay will be available to the patient through his physician if so requested. (The questionnaire and consent form used are attached)

1003536178

TABLE 1

AHH Study

Identifying information: Pure solvent with added 3 HOBP

Rosemarie H No. —

Date blood drawn 7/18/1974

Cell separation by —

Time —

Lymphocyte count —

Amount —

PHA added at —

Max. Quinine at 0.3 61.5 (a)

Calibration factor (60/(a)) = 0.9756 (b)

PHA (hrs) (c)	MC (Y/N) (d)	MC (hrs) (e)	BP (mins) (f)	Rel. Inten. (±5%) (g)	Linear Corr. (h)	Curvilinear Corr (i)	Meter Sensitivity (j)	g*j*b (k)	(g-h)*j*b (l)	(g-i)*j*b (m)	k - solv. (n)	Remarks
–	–	–	–	21	–	–	.001	.0205	–	–	–	Pure solvent
				19	–	–	.001	.0185	–	–	–	" "
				76	267	16	.001	.0741	0481	.0585	.0546	" " + 10 pico-moles 3HOBP
				76	267	16	.001	.0741	.0481	.0585	.0546	" " + 10
				75	267	16	.001	.0732	.0471	.0576	.0537	" " + 10
				46	11	10.2	.003	.1346	.1024	.1046	.1151	" " 20
				86	11.5	12.8	.003	.2517	.2180	.2142	2322	" " 40
				38	34	4.0	.01	.3707	.3376	.3317	.3512	" " 60
				52	39	43	.01	.5073	.4693	.4654	.4878	80
				66	26	0.5	.01	.6439	.6185	.6434	.6244	100

Comments:

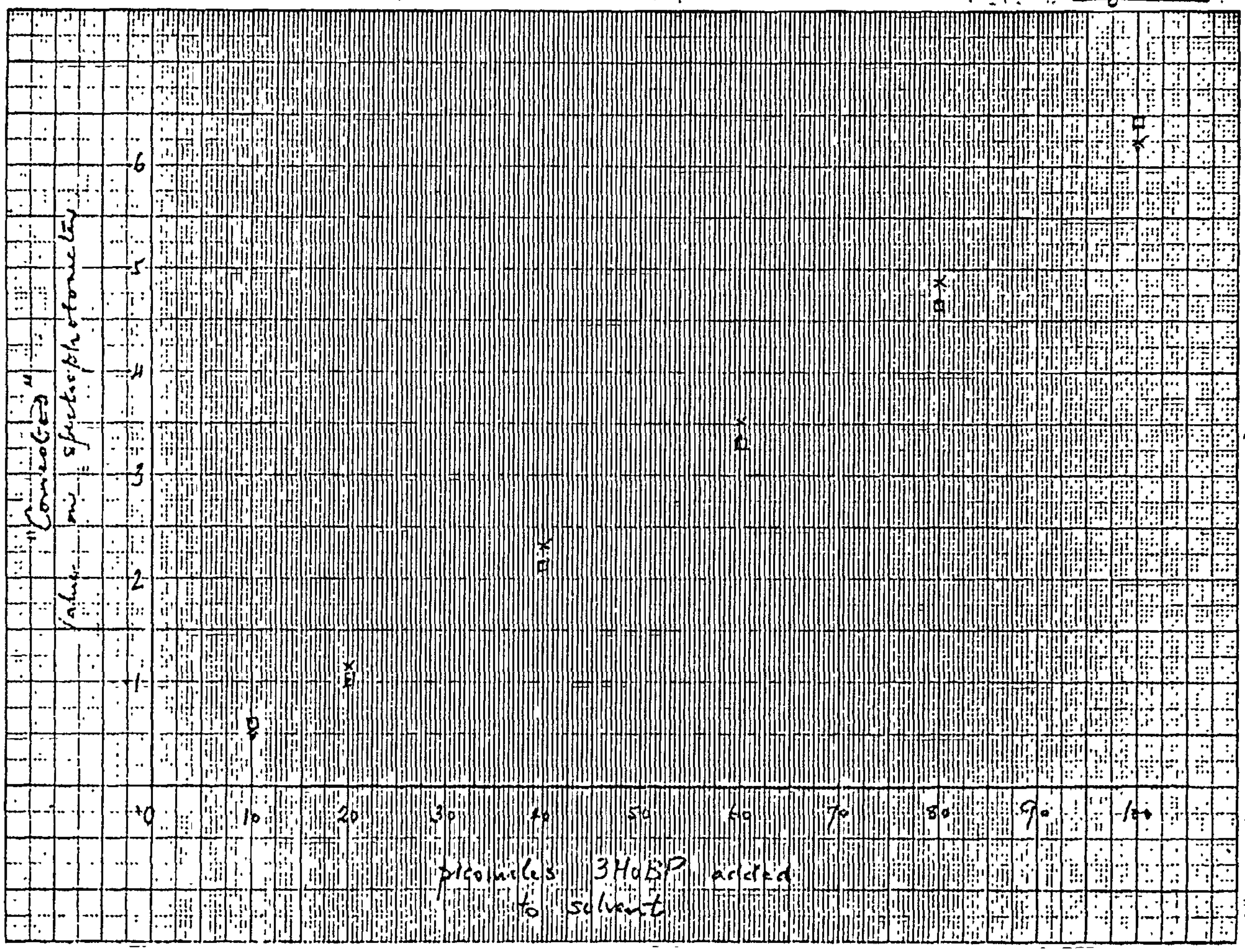
K+E 10 X 10 TO THE CENTIMETER 18 X 25 CM.
KEUFFEL & ESSER CO. MADE IN U.S.A.

46 1512

= Linear corrections

□ Curvilinear corr. × solvent corrections

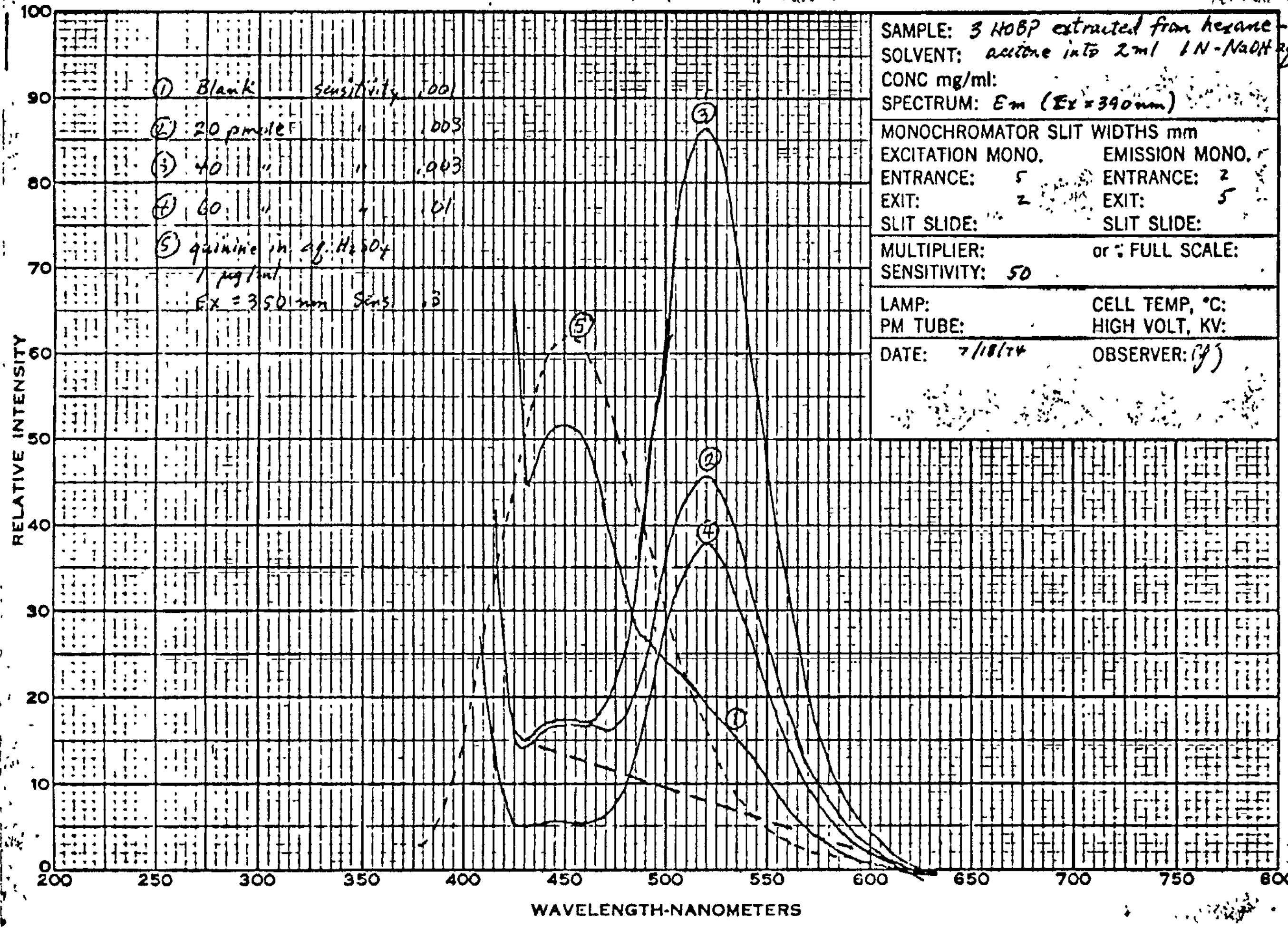
Figure 1



AMERICAN INSTRUMENT COMPANY
DIVISION OF TRAVENOL LABORATORIES, INC.
Silver Spring, Maryland 20910

Figure 2

CAT. NO. 4-8908 RECORDER PAPER



SAMPLE: 3 HOBP extracted from hexane
SOLVENT: acetone into 2ml 1N-NaOH
CONC mg/ml:
SPECTRUM: Em (Ex = 390 nm)

MONOCHROMATOR SLIT WIDTHS mm

EXCITATION MONO.		EMISSION MONO.	
ENTRANCE:	5	ENTRANCE:	2
EXIT:	2	EXIT:	5
SLIT SLIDE:		SLIT SLIDE:	

MULTIPLIER: or FULL SCALE:
SENSITIVITY: 50

LAMP: CELL TEMP, °C:
PM TUBE: HIGH VOLT, KV:

DATE: 7/18/74 OBSERVER: CJ

1003536181

Identifying information - AHH Study Greg Direnzo

Rosemarie H No. ____
Date blood drawn 8/26/1974
Time ______
Amount ______

Cell separation by Ficoll ____
Lymphocyte count ______
PHA added at ______

Max. Quinine at 0.3 71.0 (a)
Calibration factor (60/(a)) = .8451 (b)

TABLE 2

PHA (hrs) (c)	MC (Y/N) (d)	MC (hrs) (e)	BP (mins) (f)	Rel. Inten. (±5%) (g)	Linear Corr. (h)	Curvilinear Corr (i)	Meter limits (j)	g*j*b (k)	(g-h)*j*b (l)	(g-i)*j*b (m)	k-"soln" (n)	Remarks
66	N	24	60	63	19		.003	.1597	.1116		.1356	constit. level
66	N	24	60	72	32		.003	.1825	.1014		.1584	constit. level
66	N	24	0	17			.003					BP zero test – no spec. peak
66	N	24	0	11.5			.003	.0241				" — "
66	Y	24	0	10.5	12.0		.003	.0304				" — "
66	Y	24	0	13.5			.003					" — "
66	Y	24	60	73.5	17		.003	.1863	.1432		.1559	induced level
66	Y	24	60	97.5	15.7		.003	.2472	.2074		.2168	"
66	Y	24	60	33	7.7		.01	.2789	.2138		.2455	"
66	N	24	60	112.5	14.8		.003	.1078	.0702		.0837	constit. level

Comments: Assay date 8/30/1974 p.m.
using straight line correction
average constit. level = .0947 Ratio = 1.99
induced level = .1881

1003536182

Source: https://www.industrydocuments.ucsf.edu/docs/klyl0000

CURRICULUM VITAE

February, 1974

A. Personal Information:

Name: Malcolm Cecil Pike

Social Security No: REDACTED

Business Address: U.S.C. School of Medicine
Department of Community Medicine
2025 Zonal Avenue
Los Angeles, California 90033

Business Phone: 223-1379

Home Address:

REDACTED

Home Phone:

Date of Birth:

Place of Birth:

Citizenship: REDACTED

Sex:

Marital Status:

Wife's Name:

Number of Children: REDACTED

B. Education:

High School: Selborne College, East London, South Africa, R

University: University of the Witwatersrand, Johannesburg, South Africa, B. Sc., Mathematics, Pure and Applied, 1955.

University of the Witwatersrand, Johannesburg, South Africa, B.Sc. Honours, Mathematics, Pure, 1956.

Birkbeck College, London University London, England, Post Graduate studies in Statistics, 1957.

1003536183

Malcolm C. Pike, Ph.D.

B. Education: (Continued)

Cambridge University, Cambridge England, Diploma in Mathematical Statistics, 1958.

Aberdeen University, Aberdeen, Scotland, Ph.D., 1963.

Honors and Awards:

1969, Awarded the Guy Medal in bronze of the Royal Statistical Society for work on "Disease Clustering"

1972- Associate Editor for Medical Statistics and Epidemiology of Biometrics.

1972-73, Member of the Board of the British Journal of Haematology

1972-73, Member of the Board of the British Journal of Cancer.

1972-73, Member of the Council of the Royal Statistical Society.

C. Professional Background:

REDACTED

REDACTED

REDACTED

REDACTED

Malcolm C. Pike, Ph.D.

C. Professional Background: (Continued)

REDACTED

REDACTED

D. Society Memberships:

REDACTED

E. Consultantships: Developmental Research Segment, Virus Cancer Program, NCI.

Cancer Control Program, NCI.

F. Research Activities:

Research Interest: Cancer Epidemiology and Clinical Trials

Research in Progress: Epidemiology of Hodgkin's Disease, Breast Cancer, Lung Cancer. Clinical Trials in Childhood Tumors.

Grants: U.K. Department of Health and Social Security. Annual grant of $100,000 1972, 1973.

Bibliography

Articles

1. Pike, M.C.: Some numerical results for the queueing system $D/E_k/1$. J. roy. stat. Soc. B 25: 477-488, 1963.

2. Pike, M.C., Proctor, D.M., and Wyllie, J.M.: Analysis of admissions to a casualty ward. Brit. J. prev. soc. Med. 17: 172-176, 1963.

3. Blanco White, M.J. and Pike, M.C.: Appointment systems in out-patients' clinics and the effect of patients' unpunctuality. Medical Care 2: 133-145, 1964.

4. Pike, M.C. and Roe, F.J.C.: An actuarial method of analysis of an experiment in two-stage carcinogenesis. Brit. J. Cancer 17: 605-610, 1964.

5. Pike, M.C. and Alper, T.: A method for determining dose-modification factors. Brit. J. Radiol. 37: 458-462, 1964.

6. Buckton, K.E. and Pike, M.C.: Chromosome investigations on lymphocytes from irradiated patients: Effect of time in culture. Nature 202: 714-715, 1964.

7. Buckton, K.E. and Pike, M.C.: Time in culture - an important variable in studying in vivo radiation-induced chromosome damage in man. Int. J. radiation Biol. 8: 439-451, 1964.

8. Pike, M.C. and Doll, R.: Age at onset of lung cancer: Significance in relation to effect of smoking. Lancet 1: 665-668, 1965.

9. Pike, M.C.: A method of analysis of a certain class of experiments in carcinogenesis. Biometrics 22: 142-161, 1966.

10. Grant, G., Roe, F.J.C., and Pike, M.C.: Effect of neonatal thymectomy on the induction of papillomata and carcinomata by 3,4,- benzopyrene in mice. Nature 210: 603-604, 1966.

11. Medical Research Council: Treatment of acute leukaemia in adults. Brit. med. J. 1: 1383-1389, 1966.

12. Pike, M.C., Williams, E.H., and Wright, B.: Burkitt's tumour in the West Nile District of Uganda, 1961-1965. Brit. med. J. 2: 395-399, 1967.

13. Pike, M.C. and Roe, F.J.C.: Bronchi and lungs - tobacco. Raven, R.W. and Roe, F.J.C., (eds): The Prevention of Cancer. London, England, Butterworth's Publishing Company, 1967.

14. Pike, M.C., McCrae, A.W.R., and Semakula, E.: Simulium and Kaposi's sarcoma. Clifford, P., Linsell, C.A., and Timms, G.L., (eds.): Cancer in Africa. Nairobi, Kenya, East African Publishing House, 1967.

15. Garrow, J.S. and Pike, M.C.: The long-term prognosis of severe infantile malnutrition. Lancet 1: 1-4, 1967.

16. Pike, M.C., Till, M.M., Hardisty, R.M., and Doll, R.: Childhood leukaemia in greater London: A search for evidence of clustering. Brit. med. J. 3: 755-758, 1967.

17. Garrow, J.S. and Pike, M.C.: The short-term prognosis of severe primary infantile malnutrition. Br. J. Nutr. 21: 155-165, 1967.

18. Morrow, R.H., Pike, M.C., and Kisuule, A.: Survival of Burkitt's lymphoma patients in Mulago Hospital, Uganda. Brit. med. J. 4: 323-327, 1967.

19. Pike, M.C. and Smith, P.G.: Disease clustering: A generalisation of Knox's approach to the detection of space-time interactions. Biometrics 24: 541-556, 1968.

20. Grant, G.A., Carter, R.L., Roe, F.J.C., and Pike, M.C.: Effects of the neonatal injection of a carcinogen on the induction of tumours by the subsequent application to the skin of the same carcinogen. Brit. J. Cancer 22: 346-358, 1968.

21. Medical Research Council: Chronic granulocytic leukaemia: Comparison of radiotherapy and Busulphan therapy. Brit. med. J. 1: 201-208, 1968.

22. British Tuberculosis Association: Treatment of house dust allergy. Brit. med. J. 3: 774-777, 1968.

23. Williams, E.H., Spit, P., and Pike, M.C.: Further evidence of space-time clustering of Burkitt's lymphoma patients in the West Nile District of Uganda. Brit. J. Cancer 23: 235-246, 1969.

24. Uganda Buruli Group: B.C.G. vaccination against mycobacterium ulcerans infection (Buruli ulcer): First results of a trial in Uganda. Lancet 1: 111-115, 1969.

25. Arnhold, R.G. and Pike, M.C.: Patients and prescriptions: Understanding medical instructions (a study in an East African dispensary). J. trop. Paed. 14: 10-14, 1968.

26. Pike, M.C. and Morrow, R.H.: Statistical analysis of patient control studies in epidemiology. Brit. J. prev. soc. Med. 24: 42-44, 1970.

27. Pike, M.C., Morrow, R.H., Kisuule, A., and Mafigiri, J.: Burkitt's lymphoma and sickle cell trait. Brit. J. prev. soc. Med. 24: 39-41, 1970.

28. Pike, M.C.: A note on Kimball's paper "Models for the estimation of competing risks from gouped data." Biometrics 26: 579-581, 1970.

29. Bagshawe, G., Rawlins, G., Pike, M.C., and Lawler, S.: The ABO blood groups in trophoblastic neoplasia. Lancet 1: 553-556, 1970.

30. Juel-Jensen, B.E., MacCallum, F.O., MacKenzie, A.M.R., and Pike, M.C.: Treatment of zoster with idoxuridine in dimethyl sulphoxide. Brit. med. J. 4: 776-780, 1970.

31. Medical Research Council: Myelomatosis: Comparison of melphelan and cyclophosphamide therapy. Brit. med. J. 1: 640-641, 1971.

32. Morrow, R.H., Pike, M.C., Smith, P.G., Ziegler, J.L., and Kisuule, A.: Burkitt's lymphoma: A time-space cluster of cases in Bwamba County of Uganda. Brit. med. J. 2: 491-492, 1971.

33. Kinlen, L.J. and Pike, M.C.: B.C.G. vaccination and leukaemia. Lancet 2: 398-402, 1971.

34. Uganda Buruli Group: Epidemiology of Mycobacterium ulcerans infection (Buruli ulcer) at Kinyara, Uganda. Trans. roy. Soc. trop. Med. Hyg. 65: 763-775, 1971.

35. Medical Research Council: Treatment of acute lymphoblastic leukaemia: Comparison of immunotherapy (B.C.G.), intermittent methotrexate and no therapy after a 5-month intensive cytotoxic regime. Brit. med. J. 4: 189-194, 1971.

36. Bobrow, M., Pearson, P.L., Pike, M.C., and El-Alfi, O.S.: Length variation in the quinacrine-binding segment of human Y chromosomes of different sizes. Cytogenetics 10: 190-198, 1971.

37. Medical Research Council: Duration of survival of children with acute leukaemia. Brit. med. J. 4: 7-9, 1971.

38. Pike, M.C. and Morrow, R.H.: Some epidemiological problems with "EBV + malaria gives BL". Biggs, P.M., de The, G. and Payne, L.N., (eds.): Oncogenesis and Herpes Viruses, IARC Scientific Publications No. 2, 1972.

39. Kafuko G.W., Pike, M.C., et. al.: Epstein-Barr virus antibody levels in children from the West Nile District of Ugnada. Lancet I: 706-709, 1972.

40. Doll, R. and Pike, M.C.: Trends in mortality among British doctors in relation to their smoking habits. J. Roy. Coll. Physns. Lond. 6: 216-222, 1972.

41. Taylor, J.F., Smith, P.G., Bull, D., and Pike, M.C.: Kaposi's sarcoma in Uganda: Geographic and ethnic distribution. Br. J. Cancer 26: 483-497, 1972.

42. West, R.J., Graham-Pole, J., Hardisty, R.M., and Pike, M.C.: Factors in pathogenesis of central-nervous system leukaemia. Brit. med. J. 3: 311-314, 1972.

43. Baikie, A.G., Kinlen, L.J., and Pike, M.C.: Detection and assessment of case clustering in Burkitt's lymphoma and Hodgkin's disease. Brundmann, E. and Tulinius, H., (eds.): Recent Results in Cancer Research 39: 201-209, 1972.

44. Medical Research Council: Report on the first myelomatosis trial. Brit. J. Haematology 24: 123-139, 1973.

45. Till, M.M., Hardisty, R.M., and Pike, M.C.: Long survivals in acute leukaemia. Lancet 1: 534-538, 1973.

46. Campbell, A.C., Hersey, P., MacLennan, I.C.M., Kay, H.E.M., and Pike, M.C.: Immunosuppressive consequences of radiotherapy and chemotherapy in patients with acute lymphoblastic leukaemia. Brit. med. J. 2: 385-388, 1973.

47. Medical Research Council: Treatment of acute lymphoblastic leukaemia: Effect of "prophylactic" therapy against central nervous system leukaemia. Brit. med. J. 2: 381-384, 1973.

48. Brubaker, G., Geser, A., and Pike, M.C.: Burkitt's lymphoma in the North Mara District of Tanzania 1964-1970: Failure to find evidence of time-space clustering in a high risk isolated rural area. Br. J. Cancer 28: 469-472, 1973.

49. Peto, R. and Pike. M.C.: Conservatism of the approximation $(0 - E)^2/E$ in the Logrank test for survival data or tumor incidence data. Biometrics 29: 5790584, 1973.

50. Pike, M.C.: The analysis of clinical trials in leukaemia. Mathe, G., Pouillart, P., and Schwarzenberg, L., (eds.): Recent Results in Cancer Research 43: 126-132, 1973.

51. Revill, W.D.L., Pike, M.C., Morrow, R.H., and Ateng, J.: A controlled trial of the treatment of mycobacterium ulcerans infection with clofazimine with some observations on the untreated clinical course of the disease. Lancet 2: 873-877, 1973.

52. Smith, P.G. and Pike, M.C.: A note on a 'close pairs' test for space clustering. Brit. J. prev. soc. Med. (in press), 1974.

53. Smith, P.G. and Pike, M.C.: Case clustering in Hodgkin's disease: A brief review of the present position and report of current work in Oxford. Cancer Res., 34:1156-1160, 1974.

54. Pike, M.C. and Bull, D.: Knox test for space-time clustering in epidemiology. Applied Statistics (in press), 1974.

55. Pike, M.C. and Smith, P.G.: A case-control approach to examine diseases with long latent periods for evidence of contagion. Biometrics, 30, 263-279, 1974.

56. Smith, P.G. and Pike, M.C.: A case-control method of examining diseases with long latent periods. To appear in the proceedings of the IARC Conference on Cancer Epidemiology, 1973.

57. Powles, R.L., Pike, M.C., et. al.: Immunotherapy for acute myclogenous leukaemia. Brit. J. Cancer 28: 365-376, 1973.

58. Peto, R. and Pike, M.C.: Leukaemia trials. Truelove, S., (ed.): Medical Surveys and Clinical Trials. Oxford, England, Blackwell's Publishing Company, 1974.

59. Taylor, J.F., Shaw, B., Bluming, A., Briers, P., Friedman, E., Henderson, B., Horn, C., Mohan, S., and Pike, M. Tropical Myositis. Clinical and Laboratory Studies Afr. J. med. Sci., 4:409-418, 1973.

60. Gerkins, V.R., Ting, A., Menck, H.T., Casagrande, J.T., Terasaki, P.I. Pike, M.C., and Henderson, B.E. HL-A heterozygosity as a genetic marker of long-term survival. J Natl Cancer Inst 52:1909-1911, 1974.

Letters to the Editor

1. Pike, M.C.: In Dr. Baves' consulting room. American Statistician, October, 1973.
2. Pike, M.C. and Blanco White, M.J.: Outpatient waiting time. Lancet 1: 216, 1964.
3. Pike, M.C. and Blanco White, M.J.: A casualty appointment system. Lancet 1: 1104, 1964.
4. Pike, M.C.: A genetic theory of inflammatory polyarthritis. Lancet 2: 151, 1964.
5. Armitage, P., Doll, R., and Pike, M.C.: Souratic mutation. Brit. med. J. 1: 723, 1965
6. Pike, M.C.: Involutionary psychosis. Brit. J. Psychiatry 3: 551, 1965.
7. Pike, M.C.: Chemotherapy in Burkitt's tumour. Lancet 2: 856, 1966.
8. Vanier, T.M. and Pike, M.C.: Leukaemia incidence in tropical Africa. Lancet 1: 512-513, 1967.
9. Kyalwazi, S.K., Morrow, R.H., Pike, M.C., and Wright, D.H.: Treatment of Burkitt's lymphoma. Lancet 1: 1309, 1968.
10. Hamilton, P.J.S., Pike, M.C., et. al.: Absence of sickle trait in patients with tropical splenomegaly syndrome. Lancet 1: 109, 1969.
11. Pike, M.C. and Vessey, M.P.: B.C.G. and leukaemia. Lancet 2: , 1970.
12. Pike, M.C and Smith, P.G.: Epidemiology of Burkitt's lymphoma. N. Eng. J. Med. 287: 934, 1972.
13. Smith, P.G., Pike, M.C., and Kinlen, L.J.: Clustering in Hodgkin's disease. Lancet 1: 433-434, 1973.
14. Pike, M.C. and Smith, P.G.: Tonsillectomy and Hodgkin's disease. Lancet 1: 434, 1973.
15. Pike, M.C., Henderson, B.E., Casagrande, J., Smith, P.G., and Kinlen, L.J.: Infectious aspects of Hodgkin's disease. N. Eng. J. Med. 290: 341, 1974.

Computer Algorithms

1. Pike, M.C.: Random permutation. Comm. ACM 8: 445, 1965.
2. Pike, M.C.: Random normal deviate. Comm. ACM 8: 606, 1965.
3. Pike, M.C. and Hill, I.D.: Twobytwo. Computer Bulletin 9: 60, 1965.
4. Pike, M.C.: Rancomb. Computer Bulletin 9: 62, 1965.
5. Pike, M.C. and Hill, I.D.: Pseudo-random numbers. Comm. ACM 8: 605, 1965.
6. Pike, M.C. and Pixner, J.: Fibonacci search. Computer Journal 8: 147, 1965.
7. Pike, M.C. and Hill, I.D.: Algorithm of gamma function. Comm. ACM 9: 684, 1966.
8. Pike, M.C. and Hill, I.D.: Confidence interval for a ratio. Comm. ACM 9: 514, 1966.
9. Pike, M.C. and Bell, M.: Direct search. Comm. ACM 9: 684, 1966.
10. Pike, M.C. and Hill, I.D.: Incomplete beta ratio. Comm. ACM 10: 375, 1967.
11. Pike, M.C., Hill, I.D., and James, F.D.: Note on algorithm 2 - Fibonacci search and on algorithm 7 - Minx. Computer Journal 9: 414, 1967.
12. [illegible]
13. [illegible]
14. [illegible]

CURRICULUM VITAE

November 2, 1973

A. PERSONAL INFORMATION

1. Name: John Cavendish Brown
   Social Security #
2. Business Address: University of Southern California
   School of Medicine
   2025 Zonal Avenue, OCD 104
   Los Angeles, California 90033
3. Business Phone: (213) 225-3115, Extension 7-3234
4. Home Address:
5. Home Phone:
6. Birthdate:
7. Birthplace:
8. Citizenship:
9. Sex:
10. Marital Status:
11. Wife's first name:
12. Number of Children:

REDACTED

REDACTED

B. EDUCATION

1. High School: Rutlish School, Merton, England
2. College, Medical School: King's College, Strand, London
   Westminster Medical School, London
3. Internship: Surgery/Obstetrics
   Westminster Hospital, London
   1962-1963
4. Residency: Internal Medicine
   St. James's Hospital, Balham, England, 1963-1964

   Internal Medicine
   Royal Postgraduate Medical School
   Hammersmith Hospital, London,
   1964-1966

1003536193

5. Fellowships: Rheumatic Diseases and Immunology
Department of Medicine
University of California Medical Center, San Francisco, California
1966-1968

6. Honors and Awards: Chadwick Clinical Surgery Prize
Westminster Medical School, 1962

7. Licensure: General Medical Council, England
California State

8. Board Certification: Internal Medicine Boards
Royal College of Physicians, London
1965

C. PROFESSIONAL BACKGROUND

1. Academic Appointments:

1968-1971	Member of Scientific Staff Medical Research Council Rheumatism Research Unit Taplow, Berks, England
1971 - 1974	Assistant Professor of Medicine University of Southern California Clinical Immunology and Rheumatic Disease Section
1974-present	REDACTED

2. Teaching Responsibilities:

Medicine	3rd Year Basic Medical Clerkship Residents Board Review
Rheumatology	2nd Year Musculo-Skeletal Curriculum Fellow, Resident and Intern teaching Weekly ward rounds and clinics 2nd Year Medical Student elective in Musculo-Skeletal Disease
Immunology	Intern, Resident and Fellow teaching in Clinical Immunology Seminars in Cellular Immunology Medical Student Microbiology Course Reading Course in Cellular Immunology Graduate Students in Microbiology

3. Military Service:

REDACTED

1003536194

R: REDACTED MATERIAL

D. SOCIETY MEMBERSHIPS

1.
2.
3.

REDACTED

E. CONSULTANTSHIPS

1. Attending Staff, USC/Los Angeles County Medical Center
2. Consulting Rheumatologist; Martin Luther King Hospital and Charles Drew Post Graduate Medical School.

F. RESEARCH ACTIVITIES

1. Bibliography appended

2. Major areas of interest:

a) Cellular immunology related to rheumatic disease.
b) Maturation of human lymphoid tissue in relation to surface determinants on lymphocytes.
c) Studies on surface membranes of chronic lymphocytic leukemic lymphocytes.
d) Cell mediated immunity to RNA tumor viruses

3. Book

Title:	Pratical Rheumatology
Publisher:	W. B. Saunders
Authors:	J. C. Brown and D. M. Forrester

Scheduled for publication in 1974

1003536195

R: REDACTED MATERIAL

D. SOCIETY MEMBERSHIPS

1.
2.
3.

REDACTED

E. CONSULTANTSHIPS

Attending Staff, USC/Los Angeles County Medical Center

F. RESEARCH ACTIVITIES

1. Bibliography appended

2. Major areas of interest:

   a) Cellular immunology related to rheumatic disease.
   b) Maturation of human lymphoid tissue in relation to surface determinants on lymphocytes.
   c) Studies on surface membranes of chronic lymphocytic leukemic lymphocytes.

3. Research now in progress:

   a) Synthesis of immunoglobulin determinants on the surface of human lymphocytes.
   b) Development and maturation of human lymphoid tissue.
   c) Clinical Immunology - Immunologic profile of chronic active hepatitis, primary biliary cirrhosis, leprosy and sarcoidosis.

4. Book

Title:	Practical Rheumatology
Publisher:	W. B. Saunders
Authors:	J.C. Brown and D.M. Forrester

Scheduled for publication in 1974

1003536196

BIBLIOGRAPHY

John Cavendish Brown, M.D., M.B., M.R.C.P.

1. Graham, R., Brown, J.C., Graham, O.: A controlled trial of hydroxytoluic acid. VIth European Congress of Rheumatology Lisbon, 1967.

2. Brown, J.C., Epstein, W.V.: The inhibition of antibody forming cells by human rheumatoid factor. IV Pan American Congress of Rheumatology, Mexico City, 1967.

3. Brown, J.C.: Late maturity onset rheumatic syndromes, In: Symposium on Clinical Rheumatology. The Arthritis Foundation Stanford University, California 1968.

4. Brown, J.C., Epstein, W.V.: The specificity of inhibition of antibody-producing cells by human rheumatoid factor. Presented at the interim meeting of the American Rheumatism Association. Abstract Arthritis Rheumatism, XI, 96, 1968.

5. Brown, J.C., Epstein, W.V.: Influence of human rheumatoid factor on numbers of antibody producing cells. Arthr. Rheum. XII,L, 1968.

6. Holborow, E.J., Schwab, J.H. and Brown, J.C.: Capacity of isolated cell walls from Group A streptocci to induce auto-immune processes. Folia Allergologica, 16:287, 1969.

7. Brown, J.C., Epstein, W.V.: Current knowledge of pathogenetic mechanisms of rheumatic disorders. Postgrad. Med., 45:78, 1969.

8. Brown, J.C., Schwab, J.H. and Holborow, E.J.: Distribution of haemocyanin and of immunogenic and nonimmunogenic human gamma globulin within draining auricular lymph nodes of guinea pigs. British Society of Immunology, May, 1969.

9. Brown, J.C., Holborow, E.J., Schwab, J.H.: The effect of rheumatoid factor on uptake of aggregated human gamma globulin in lymphoid tissue. XII International Congress of Rheumatology, Prague, 1969.

10. Holborow, E.J., Schwab, J.H., Brown, J.C.: Capacity of isolated cell walls from Group A streptococci to induce autoimmune processes. Folia Allergologica, 16:287, 1969.

11. Brown, J.C., Schwab, J.H. and Holborow, E.J.: The uptake of immunoglobulin and immune complexes in lymphoid tissue. Immunology, 19:401, 1970.

12. Brown, J.C., De Jesus, D.G., Holborow, E.J., Harris, G.: Lymphocyte-mediated transport of aggregated human gamma globulin into germinal centre areas of normal mouse spleen. Vol. 228, 5269:367, 1970. Nature.

13. Papamichail, M., Brown, J.C. and Holborow, E.J.: Immunoglobulins on the surface of human lymphocytes. Lancet, 2:850, 1971.

14. Brown, J.C., De Jesus, D.G. and Holborow, E.J.: The inability of NZB and B/W hybrid mice to localize altered IgG splenic germinal centres. Presented at the Meeting of the American Rheumatism Association, January 1971, Washington, D.C. and VIIth European Rheumatology Congress, Brighton, 1971.

15. Greenwood, B.M., Brown, J.C., De Jesus, D.G. and Holborow, E.J.: Immunosuppression in murine malaria. II. The effect on reticuloendothelial and germinal centre function. Clin. & Exp. Immunol., 9:345, 1971.

16. De Jesus, D.G., Holborow, E.J. and Brown, J.C.: A defect of B lymphocyte transport of aggregated HGG into germinal centers in NZB and NZB/NZW f1 hybrid mice. Clin. Exp. Immunol. 11:507, 1972.

17. Brown, J.C., Harris, G., Papamichail, Slijvic, V., Holborow, E. J.: The localization of aggregated human gamma globulin in the spleens of normal mice. Immunology 24:955, 1973.

18. Nies, K.M., Oberlin, M.A., Brown, J.C., Halpern, M.S.: Immunoglobulin synthesis by normal and leukemic human peripheral blood lymphocytes. J. Immunol. Vol. 111, 4:1236, 1973.

19. Baros, M.P., Nies, K.M., Brown, J.C., Acton, R.T., Baker, J.A. Parker, J.W., Lukes, R.J.: Leukemic reticuloendotheliosis, functional and morphologic studies (submitted for publication) 1973.

20. Nies, K.M., Brown, J.C., Dubois, E.L., Quismorio, F.P., Friou, G.J., Terasaki, P.I.: HL-A antigens and lymphocytotoxic antibodies in SLE. Annual Scientific Sessions, American Rheumatism Association, 1973. (Abstract in Arthritis & Rheumatism. Detailed paper submitted for publication.)

R: REDACTED MATERIAL

Date: August 2, 1974

CURRICULUM VITAE

A. Personal Information:

Name in Full	Robert Julian Gordon, Ph.D.
Business Address	Department of Pathology USC School of Medicine 1840 North Soto Street Los Angeles, California 90032
Business Phone	226-2326
Home Address	
Home Phone	REDACTED
Date of Birth	
Place of Birth	
Citizenship	
Marital Status	
Spouse's First Name	
Number of Children	
Social Security Number	REDACTED

B. Education:

High School	North Hollywood High School.
University	University of California, Los Angeles B.S. Chemistry, 1947 Ph.D. Organic Chemistry, 1952
Honors	Sigma Xi

C. Professional Background:

Academic Appointments:

REDACTED REDACTED

Specific teaching responsibilities:

REDACTED

Military service:

REDACTED REDACTED

1003536199

Other employment or activity

REDACTED

REDACTED

REDACTED

REDACTED

REDACTED

Biography

American Men of Science (Physical Sciences)

D. Society Memberships:

National

REDACTED

E. Consultantships:

U.S. Environmental Protection Agency, Chaple Hill, North Carolina

Pacific Environmental Services, Santa Monica, California

F. <u>Research Activities</u>:

Bibliography Appended.

Research in Progress:

Study of air pollution and other environmental factors in relation to etiology and epidemiology of human cancer.

1003536201

BIBLIOGRAPHY

1. Gordon, R. J., Moore, R. J., and Muller, C. E.
Aromatic types in heavily cracked gas oil fraction. Combined use of ultraviolet and mass spectrometry.
Anal. Chem. 30: 1221-1224, 1958.

2. Gordon, R. J. and Eiffert, R. C.
Analytical applications of near infrared spectroscopy.
In Vth World Petroleum Cong., New York. Section 5: 13-20, 1959.

3. Gordon, R. J. and Heath, A. E.
Photochemical reaction of ethylene and nitrogen dioxide.
Western regional ACS meeting, Los Angeles, California, November 1965.

4. Gordon, R. J.
Photochemical measurement of solar ultraviolet radiation.
National air pollution control association meeting, San Francisco, June 1966.

5. Romanovsky, J. C., Ingels, R. M and Gordon, R. J.
Smog effects with nitrogen oxides-hydrocarbon mixtures.
J. Air Pollu. Contr. Ass. 17: 454-459, 1967.

6. Gordon, R. J. and Bonamassa, F.
UV sunlight and smog effects in Los Angeles.
Western regional ACS meeting, Los Angeles, California, October 1967.

7. Gordon, R. J.
Pilot study of ultraviolet radiation in Los Angeles, 1965.
In Public Health Service Publication No. 999-AP38, Photochemical Measurements (Nader JS, ed.), chapt. 3, 1967.

8. Gordon, R. J., Mayrsohn, H. and Ingels, R. M.
C_2 -C_5 hydrocarbons in the Los Angeles Atmosphere.
Envir. Sci. Tech. 2: 1117-1120, 1968.

9. Freeman, A. E., Price, P. J., Gordon, R. J., Bryan, R. J. Gilden, R. V., Kelloff, G. J. and Huebner, R. J.
Transformation of rat and hamster embryo cells by extracts of city smog.
Proc. Nat. Acad. Sci., Wash. 68: 445-449, 1971.

10. Rhim, J. S., Cho, H. Y., Rabstein, L., Gordon, R. J., Bryan, R. J., Gardner, M. B. and Huebner, R. J.
Transformation of mouse cells infected with AKR leukaemia virus induced by smog extracts.
Nature 234: 103-107, 1972.

BIBLIOGRAPHY (continued)

11. Gordon, R. J., Bryan, R. J., Rhim, J. S., Demoise, C., Wolford, R. G., Freeman, A. E. and Huebner, R. J.
Transformation of rat and mouse embryo cells by a new class of carcinogenic compounds isolated from particles in city air.
Int. J. Cancer 12: 223-232, 1973.

12. Gordon, R. J. and Bryan, R. J.
Ammonium nitrate in airborne particles in Los Angeles.
Envir. Sci. Tech. 7: 645-647, 1973.

13. Rhim, J. S., Gordon, R. J., Bryan, R. J. and Huebner, R. J.
Transformation of mouse cells infected with AKR leukemia virus by benzene extract fractions of city air particles.
Int. J. Cancer 12: 485-492, 1973.

14. Gordon, R. J. and Bryan, R. J.
Patterns in airborne polynuclear hydrocarbon concentrations at four Los Angeles sites.
Envir. Sci. Tech. 7: 1050-1053, 1973.

15. Gordon, R. J.
Solvent selection in extraction of airborne particulate matter.
Atmos. Envir. 8: 189-191, 1974.

BIBLIOGRAPHY (continued)

Books

Gordon, R. J.
Photochemical smog, chapt. 2.4a; Carcinogens, chapt. 3.3; Hydrocarbons, chapt. 3.7; Aldehyde sensors, chapt. 4.4; Carbon monoxide sensors, chapt. 4.7; Hydrocarbon sensors, chapt. 4.10; Nitrogen oxide sensors, chapt. 4.13; Total oxidant sensors, chapt. 4.20.
In: Environmental Engineer's Handbook (Liptak, B., ed), Chilton Book Company, in press.

BIBLIOGRAPHY (continued)

<u>Miscellany</u>

Patent:

Moore, R. J., Handschy, J. and Gordon, R. J.
Alkylation process, U.S. 3050453, (August 21, 1962)

STUDY OF RELATIONSHIP BETWEEN SUSCEPTIBILITY TO VARIOUS CANCERS AND ARYL HYDROCARBON HYDROXYLASE INDUCIBILITY

PROGRESS REPORT

September 13, 1974

Period covered: July 1, 1974 - October 31, 1974

Summary:

The core problem of this study still remains to be solved as of this date, viz. the establishment of a repeatable test. Some progress has been made in this direction, both at Dr. Kouri's laboratory and at our laboratory, but the firm, positive statement that we must be able to make before we can really begin epidemiological work, cannot yet be made.

Details of where we are in the laboratory testing here at USC are given below. In summary, the chemistry (spectrophotomer) part of the test has been satisfactorily established but the variability between split-samples of bloods run in parallel is still too great for comfort.

Dr. Kouri appears to be able to run split-samples in parallel at an acceptable level of variability, but the air-freighting of whole blood to Bethesda is not working satisfactorily (blood arriving too cold, failing to separate properly, and reduced stimulation with PHA). We have thus not yet been able to test whether day-to-day variation in

Dr. Kouri's laboratory is tight enough. (The initial collaboration between ourselves and Dr. Kouri involved cell separation, PHA stimulation, and addition of 3MC at our laboratory before sending to Bethesda for BP conversion testing: this was abandoned when it became clear that the BP conversion measurement was not the part of the assay giving trouble.)

Arrangements for collecting blood from "cases" and "controls" are set up and we envisage little or no trouble supplying the laboratories with samples once the test system is firmly established: we are currently sending 12 30 ml samples to Dr. Kouri each week testing for repeatability.

Details of Progress at USC

(a) Spectrophotomer assay for 3-Hydroxybenzo(a)pyrene (3HOBP)

This is begun by suspending the stimulated medium-free cells in one ml of a mixture of Tris buffer and $MgCl_2$, adding 100 μg benzo(a)pyrene in 50 μl acetone, and incubating at 37°C for 45-60 minutes (a blank reagent control is run without incubation). After incubation the mixture is quenched by shaking with a mixture of 1 ml acetone/ 3.25 ml n-hexane, and after centrifugation, 3 ml of the upper organic layer are withdrawn and extracted with 2 ml 1N-NaOH (aqueous). The NaOH layer is analyzed by fluorescence with an excitation wavelength of 390 nm.

We began by putting the 3HOBP through the last steps (starting with a hexane/acetone solution), using the reference material supplied by Dr. Kouri. We worked in a room under orange light (nil below 450 nm) with spectrograde solvents and high quality water, but recoveries were still erratic until we used a nitrogen purge to reduce oxidative degradation. By flushing tubes and spectrometer cuvettes and purging solvents with nitrogen we got good repeatability and linear response up to 100 p mole 3HOBP in 2 ml NaOH (see Table 1 and Figure 1: the 3 separate sets of points plotted refer to 3 different methods of 'correcting' for 'background'). Examples of the emission spectra are shown in Figure 2. Our solvents give blanks in general similar to the one shown, but there

is variation from one sample to another which would be important for samples in the range found for constitutive level AHH activities. We propose that an approximation to the solvent contribution be made by using the conventional baseline spectral correction method. In this case a line is drawn from the minimum in the 430-470 nm region tangent to the curve around 600 nm. The vertical distance between this line and the maximum at 520 nm is read as the corrected emission intensity. The example for trace 3 in Figure 2 gives a correction of .024 (= 8*.003) as against .019 (=19*.001) for correction using the solvent trace. We have also tried curvilinear corrections but these gave no improvement over the linear.

Our next step was to spike various PHA stimulated cell samples, incubated without benzo(a)pyrene, after the hexane-acetone quench, with known additions of 3HOBP. The recoveries were consistent and repeatable but not as precise as the previous series.

Further 'purely chemistry' testing appeared unwarranted by this stage as we were experiencing major biological variation.

(b) Experience with actual complete test system

Table 2 shows the stage of repeatability we have reached with the complete test system, i.e., Ficoll separation, 66 hours PHA, 24 hours 3MC, 60 minutes BP.

This sample of blood was split 10 ways -- all subsamples of 4 million lymphocytes before PHA stimulation. All subsamples had PHA added for 66 hours, then 5 had 3MC added ("induced") for 24 hours and 5 were similarly treated without 3MC ("uninduced" or "constitutive"). For each set of 5, 3 were incubated with BP for 60 minutes and 2 for zero minutes. The repeatability of these zero time controls was: for no MC, 8 and 11; for MC, 10.5 and 13.5 (scale units). Using these as corrections the constitutive levels were .084, .136, .158 and the induced levels .156, .217, .249 (arbitrary units). Using our straightline correction, this was improved to .072, .101, .112 and .143, .207, .214 giving an inducibility factor of .188/.095=1.99.

We are not satisfied with this degree of variation and are trying a number of ways to reduce it. Essentially the problem appears to be non-uniformity of PHA stimulation in separate tubes and it may be that the only solution will be to do many tubes and average the results. The quantity of blood reasonable to draw from a patient will mean that if this does turn out to be the case we will have to find micromethods of detecting 3HOBP. We will look into this possibility if necessary as soon as we have stabilized our laboratory procedures to the point where we are not reducing our variability any further. At the present time the sheer amount of manipulation required to do the test still makes it reasonable to assume that we will continue to improve for at least a few more weeks by experience alone.

April 24, 1974

J. H. Kreisher, Ph.D.
Associate Research Director
The Council for Tobacco Research - USA, Inc.
110 East 59th Street
New York, N.Y. 10022

Dear John,

I am writing following up on Henderson's telephone call of last week.

Kouri is now relatively happy with his test and has instructed our immunologist (Dr. John Brown) how to do the test procedure up to the stage before adding BP, i.e., we separate the lymphocytes, add PHA and PW, then MC (or not) and finally freeze the samples for shipment to Kouri. The first batch of test sera goes off next week. These test sera are checking our technique, and variables such as numbers of cells in culture, amount of nitrogen required, glass v plastic, method of separation of cells. The results of these sera and others to be tested in the next 3-6 weeks should enable us to settle on a production method.

We trust therefore that we will start our field studies in early June.

We have received the go ahead to study AHH inducibility in patients from the six hospitals in Los Angeles County whose cooperation we had requested. The number of new patients diagnosed in 1972 with cancers of interest re AHH is given in Attachment #1. Initially we will concentrate on cancer of the lung (for obvious reaons), breast and pharynx (we have ongoing studies of these two sites), and then move on to studying the other sites. Detailed plans are given in Attachments #2 and #3.

Financial help from your Council would be of great assistance to us in completing these studies. We would therefore like to request from them funds for one year (in the first instance) as per

COPY

1003536211

Attachment #5. With these funds we will be able to process about 20 samples per week; projected completion dates of studies given on Attachments #2 and #3 are based on this requested level of funding.

Henderson mentioned that you may be able to consider funding this work on a monthly basis pending your Council's next relevant meeting. I would be grateful for your advice on how to proceed with this funding request.

Yours sincerely,

Malcolm C. Pike, Ph.D.
Professor, Community ;Medicine
and Pediatrics

P.S. The chemist here (Dr. Robert Gordon) and I have been doing some thinking about the possible (probable?) mechanism of AHH inducibility and cancer induction (see Attachment #4) and have come to the conclusion that measuring many more metabolites of BP might be very informative (method given in Science, 12 April 1974, 169-171). What are the possibilities of funding us to do this? Incidentally, if Kouri gets overwhelmed at MBA, Gordon sees no problem in completing Kouri's test here in L.A.

mcp/ml
Encl.

COPY

Attachment #1

#1972 Male Cases from CSP File by Site

Site	Hospital: USC/LAC	VA Wadsworth	VA Sepulveda	VA Long Beach	Kaiser Sunset	Harbor General	Total
Lung	127	55	57	82	57	46	424
Colon	15	15	17	19	48	11	125
Bladder	17	16	24	29	37	9	132
Esophagus	17	11	4	7	3	6	48
Mouth	24	7	2	15	12	1	61
Pharynx	15	11	2	16	7	3	54
Lip	4	11	1	6	3	0	25
Larynx	19	21	2	16	8	9	75

#1972 Female Cases from CSP File by Site

Site	Hospital: USC/LAC	Kaiser Sunset	Harbor General	Total
Lung	38	31	14	83
Breast	107	134	45	286
Colon	39	29	7	75
Bladder	13	8	1	22
Esophagus	9	4	3	16
Mouth	10	7	3	20
Pharynx	3	1	5	9
Larynx	5	4	2	11

Attachment #2

Lung Cancer

1. Hospital Study

We will interview 100 lung cancer patients (and take 15 ml blood sample) using a detailed questionnaire (Attachment #2.1, based on the Comprehensive Tobacco Questionnaire of the American Health Foundation and also including details of current medication). The patients will be interviewed soon after admission (when diagnosis may possibly still be in doubt). Clinical and pathological details on the patients will be obtained from hospital records.

100 matched hospital "controls" will also be interviewed in the same manner as the lung cancer patients. They will be sex, race, age matched within same 5-year age group, and will be drawn from the same hospital population. The controls will be chosen as the next new suitable patient entering the hospital with a non-neoplastic, non-respiratory disease.

We will analyze the results of the study in terms of smoking habits, tumor cell type, age and AHH inducibility (and base levels).

Adenocarcinoma of the lung is not thought to be related to cigarette smoking, but the relation of this cell type to AHH inducibility is of definite interest. Out of 100 lung cancer cases we expect only a few (10-20) adenocarcinomas, we will increase this number to 50 by selectively interviewing this type of case.

This study is projected to be completed by December, 1974.

2. Leisure World Study

We will test 100 long-term cigarette smokers over age 75 without cancer from the residents of "Leisure World", a retirement community south of Los Angeles County.

This study is projected to be completed by December, 1974.

The need for "controls" for this study will depend on whether we find an age and social class effect in our "controls" from the Hospital Study.

3. Environmental and Occupational Study

Depending on the answers to our questions on the measurement of AHH "base levels" (see attachment #4) we will look at these levels in high risk to lung cancer groups. In particular, we will look at the levels in persons exposed occupationally or at home to high levels of airborne PAH, and to persons in high risk to lung cancer trades (e.g. printers, painters, asbestos workers). This could give us a model for interaction between say smoking and asbestos exposure.

This study obviously cannot as yet be projected as regards time to completion.

Attachment #2 (cont'd.)

4. Ethnic Group Distribution Study

If the hospital study confirms the Kellerman, et al findings, then we will test in the first instance 100 healthy, young Mexican-Americans and 100 healthy young Anglo-whites to see if there is a difference in AHH levels in the two groups. Such a difference may be partly responsible for the lower rates of lung cancer in the Mexican-Americans in Los Angeles County.

This study is projected to be completed by May, 1975.

1003536215

Attachment #3

Cancer at Other Sites

It is of obvious interest to check on the relationship between AHH and tumors of sites other than lung. In particular those sites that have been connected with smoking or with PAH induction in animals.

We will, therefore, interview and collect blood samples from 50 cases with cancer at each of the following sites: breast, pharynx, bladder, esophagus, larynx, colon, mouth, lip.

The breast cancer patients will be prevalent cases we have already interviewed for another study. They will be able to be collected by September, 1974.

The pharynx cancer patients will consist of both prevalent cases (about 25) we have already interviewed for another study and new cases reported to the hospitals given in Attachment #1.

Patients with tumors at one of the other sites will be obtained from these same six hospitals.

All these studies should be completed by May 1975.

1003536216

Attachment #4

Basic Understanding of AHH Behavior

Further basic understanding of the mechanism of the correlation between AHH inducibility and lung cancer would help greatly to shape our approach to epidemiological studies. We feel particularly ignorant in this area.

The first question we would like an answer to is whether a person's base level of AHH activity in lymphocytes, i.e. no MC added to test, is affected by smoking or breathing PAH laden air? I.e., if I don't smoke for a week and the AHH activity in my lymphocytes is measured is it lower than it would be if I had smoked two packs a day for the week? If the answer is 'no', is it 'yes' for lung tissue AHH activity? The answer to the latter must (?) be 'yes'.

This first question is easy to answer and will do so in the next few months (unless we find that the answer is already known).

If we need to look at lung tissue AHH activity, could you please suggest to us how to do this.

The second question we have is why, if all BP is broken down through the same metabolic pathways independent (?) of AHH inducibility level is high AHH inducibility associated with cancer induction? I.e., if a person with high AHH inducibility simply converts the BP quicker but in no greater absolute amounts, why is he at higher risk? We are trying to get an understanding of this through discussions with local enzymologists but would welcome advice and/or information.

The third question is what drugs affect AHH levels? Antitumor agents? Barbiturates? What else? How do they affect levels? Can we use patients on these drugs in studies? It is obvious that we can answer some of these questions (as for question #1) but again we welcome advice and/or information.

Attachment #5

First Year Budget

	Cost	Sub-Total
Equipment		
Double viewing tube for Zeiss RA microscope	$± 500.	
Coulter counter (Model ZB1)	5,874.	$ 6,374.
Supplies		
Biologicals	$ 2,000.	
Chemicals	1,500.	
Glassware and disposables	3,000.	
Computing	1,000.	
Equipment maintenance	700.	
Phones	600.	
Incidentals (office supplies, xerox, postage, printing charges, reprints, etc.)	500.	
Airfreight	1,000.	$ 10,300.
Salaries		
Technician (Tech III)	11,000.	
Nurse/interviewer	13,700.	
Secretary/clerk (½ time)	4,000.	$ 28,700.
Travel		
Local (to hospitals, etc.)	2,000.	
Meetings, etc.	1,500.	$ 3,500.
Fringe benefits (12% of salaries and wages)		$ 3,444.
University overhead (15% of salaries and wages)		$ 6,375.
Total		$ 58,693.

1003536218

CONFIDENTIAL

UNIVERSITY OF SOUTHERN CALIFORNIA
SCHOOL OF MEDICINE
A H H STUDY #1

Code # AHH1/__________

*Participant's Name: ____________________

*Address: ________________ City ________________ Zip ________

*Phone: ______________

*Married: Yes/No

*Next of Kin: ________________

Blood Sample Drawn: Time: ________________

Date: ________________

Is patient aware of any diagnosis as of this date: Yes/No
(If yes - what diagnosis; ask Ward Nurse before interview):

____

*Diagnosis: ________________________ (Confirmed: Yes/No) 1003536219

(Attach pathology reports to back of form)

*Date of diagnosis: ________________

Give brief hospital history from Date of Diagnosis to Date of
Blood Sample Drawn:

____

____

____

CSP # (if any) ______________.

*Information to be obtained from patient and/or patient's medical chart.

A. Are you currently taking any medications? (Antibiotics, analgesics, depressants, stimulants, hormones - incl. insulin, steroids, thyroid).

Yes ______ No ______

B. If yes, drugs taken within the past 24 hours:

Drug	Dosage	Exact time taken

C. Drugs taken within the past 30 days:

Drug	Dosage	Frequency

D. Have you ever had radiotherapy?

Yes ______ No ______

If yes, when: ______________________________

1003536220

USC School of Medicine
A H H #1

E. How much smoking did you do last week?

	Cigarettes	Cigars	Pipes
Today:			
1 day ago:			
2 days ago:			
3 days ago:			
4 days ago:			
5 days ago:			
6 days ago:			

F. When did you smoke your last cigarette/cigar/pipe? ____________

____

1003536221

Code # AHH1/ (1-9) | A | H | H | 1 | / | | | | |

Card #1 (10) | 1 |

1. Hospital ______________________ (11-12) | | |

*2. Medical Record # ______________________

*3. Date of diagnosis ____/____/____ (13-18) | | | | | | |
Month Day Year

4. Interviewer ______________________ (19) | |

5. Date of Interview ____/____/____ (20-25) | | | | | | |
Month Day Year

6. Sex: 1 Male 2 Female (26) | |

7. Date of Birth ____/____/____ (27-32) | | | | | | |
Month Day Year

8. Religion (Specify) ______________________

1 Protestant
2 Catholic
3 Jewish
4 Other ______________________ (33) | |

9. Place of Birth ______________________
Town/City Country

1 U.S.-urban
2 U.S.-rural
3 Foreign-urban
4 Foreign-rural
5 U.S.-D.K.
6 Foreign-D.K. (34) | |

10. Age entered U.S. - (If foreign born) ________ (35-36) | | |

*11. Race: 1 Caucasian
2 Black
3 Oriental
4 Mexican-American
5 Other ______________________ (37) | |

1003536222

12. Education (Highest school attended) (38) ☐

1 Graduate Professional Training
2 College Graduate
3 Partial College
4 High School Graduate
5 Partial High School
6 Junior High School (7-9th Grade)
7 Less than 7th Grade

13. Present Occupation (Specify) ____________ (39) ☐

1 Professional
2 Business Executive
3 Administr. Personnel (Sm. Business Owners)
4 Clerical/Sales
5 Skilled
6 Semi-skilled
7 Unskilled
8 Retired/Unemployed
9 Housewife

14. Former Occupation (If retired or unemployed) (Specify) - (40) ☐

15. Husband's Occupation (If married female) (Specify) - (41) ☐

16. Occupational Exposure ____________ (42) ☐

16A. Diagnosis:

Site: (43-46)				
Hist: (47-50)				

Tobacco Usage

Code # AHH1/ (1-9) A H H 1 /

Card #2 (10) 2

17. Type of Tobacco Ever Smoked

1 Cigarette only
2 Cigarette and cigar
3 Cigarette and pipe
4 Cigar only
5 Cigar and pipe
6 Pipe only
7 All three
8 Never smoked

(11)

18. Chewing Tobacco

1 Ever chewed
2 Never chewed

(12)

19. Snuff

1 By nose
2 By mouth
3 By nose and mouth
4 Never

(13)

Cigarettes (If Q 17 answer is 1,2,3, or 7)

20. Age began smoking cigarettes ____________ (14-15)

21. Do you still smoke cigarettes?

16. 1 Yes (Present smoker) 2 No (Ex-smoker) (16) –

22. When did you stop smoking? (If stopped) ____________

Specify date if known

Years and months since stopping. (17-20)

23. Why did you stop? (If stopped) ____________ (21)

1003536224

Filtered		Unfiltered
1 Winston	16 Old Gold	31 Pall Mall
2 Marlboro	17 Kool	32 Camel
3 Pall Mall	18 Lark	33 Raleigh
4 Salem	19 Paxton	34 Chesterfield
5 Kent	20 Parliament	35 Lucky Strike
6 Tareyton	21 Virginia Slims	36 Philip Morris
7 Camel	22 Silva Thins	37 Old Gold
8 True	23 Carlton	38 Kool
9 Benson & H.	24 Marvels	39 Mixed-Unfiltered
10 Viceroy	25 Spring	40 Other-Unfiltered
11 L.& M.	26 Doral	
12 Chesterfield	27 Montclair	99 Mixed-filtered and unfiltered brands
13 Newport	28 Mixed-Filtered	
14 Lucky Strike	29 Other-Filtered	
15 Philip Morris	30 Vantage	00 Never smoked cigarettes

24. Most recent brand ________ (22-23)
    a. Size ________ (24)
    b. Number of years ________ (25-26)
    c. Average no./day ________ (27-28)
25. Previous brand ________ (29-30)
    a. Size ________ (31)
    b. Number of years ________ (32-33)
    c. Average no./day ________ (34-35)
26. Previous brand ________ (36-37)
    a. Size ________ (38)
    b. Number of years ________ (39-40)
    c. Average no./day ________ (41-42)
27. Previous brand ________ (43-44)
    a. Size ________ (45)
    b. Number of years ________ (46-47)
    c. Average no./day ________ (48-49)

Cigarette Size
1 Regular
2 King
3 100 mm
4 Various

1003536225

28. Number of years smoked cigarettes ____________ (50-51)

29. Number of years smoked filters ____________ (52-53)

30. Number of years smoked non-filters ____________ (54-55)

31. Inhalation (Cigarettes - last brand smoked) (56)

1 Deeply into chest
2 Partly into chest
3 Back to throat
4 Inhale DK how deeply
5 Do not inhale

32. Cigarette length smoked (Last brand smoked) (57)

1 All
2 3/4
3 1/2
4 1/4
5 DK

33. Use of cigarette holders with non-filters (Last brand smoked) (58)

1 Always 2 Sometimes 3 Never

Cigars (If Q 17 answer is 2,4,5, or 7)

34. Age began smoking cigars ____________ (59-60)

35. Do you still smoke cigars? (61)

1 Yes (Present smoker) 2 No (Ex-smoker)

36. When did you stop smoking cigars? (If stopped) ____________

____

Specify date if known

Years and months since stopping. (62-65)

37. Why did you stop? (If stopped) ____________ (66)

____

38. Number of years smoked cigars ____________ (67-68)

39. Average number of cigars smoked per day ____________ (69-70)

40. Inhalation (Cigars) (71)

1 Deeply into chest
2 Partly into chest
3 Back to throat
4 Inhalation DK how deeply
5 Do not inhale

1003536226

Pipes (If Q 17 answer is 3,5,6, or 7)

Code # AHH1/ ____________ (1-9) A H H 1 /

Card #3 (10) 3

41. Age began smoking pipes ____________ (11-12)

42. Do you still smoke pipes?

1 Yes (Present smoker) 2 No (Ex-smoker) (13)

43. When did you stop smoking pipes? (If stopped) ____________

Specify date if known

Years and months since stopping. (14-17)

44. Why did you stop? (If stopped) ____________ (18)

45. Number of years pipe smoking ____________ (19-20)

46. Average number of pipefuls smoked per day ____________ (21-22)

47. Inhalation (Pipes)

1 Deeply into chest
2 Partly into chest
3 Back to throat
4 Inhalation DK how deeply
5 Do not inhale

(23)

Chewing Tobacco (If Q 18 answer is 1)

48. When did you stop chewing tobacco? (If stopped) ____________

Specify date if known

Years and months since stopping. (24-27)

49. Why did you stop? (If stopped) ____________ (28)

50. Number of years chewing tobacco ____________ (29-30)

51. Frequency of Use (31)

1 Once a day or more
2 Once a week or less than once a day
3 Less than once a week

1003536227

Snuff (If Q 19 answer is 1,2, or 3)

52. When did you stop using snuff? (If stopped) ________________

____
Specify date if known

Years and months since stopping. (32-35) [| | |]

53. Why did you stop? (If stopped) ________________________ (36) [ ]

____

54. Number of years of snuff use ____________ (37-38) [|]

55. Frequency (39) [ ]

1 Once a day or more
2 Once a week or less than once a day
3 Less than once a week

TELEPHONE:
CAPITOL 5-1511

UNIVERSITY OF SOUTHERN CALIFORNIA
SCHOOL OF MEDICINE
DEPARTMENT OF PATHOLOGY
2025 ZONAL AVENUE
LOS ANGELES, CALIFORNIA 90033

PATHOLOGY

PATIENT CONSENT FORM

____
Date

We are conducting a study in an effort to learn more about the influence of certain enzyme functions in causing disease. We would like your participation in this study. To do so requires completing a questionnaire regarding your residential and occupational history, and tobacco usage. In addition, it involves withdrawing a small blood sample from your arm by hypodermic needle. Your arm may be sore for a short period of time. Any questions you may have will be answered as completely as possible.

By signing below, I consent to participate in the study. I understand that this is an effort to increase man's knowledge of diseases, and that any information obtained from me will be kept strictly confidential. I realize that I have the right to deny or withdraw my consent to participate at any time.

____
Signature of Patient

____
Signature Witnessed
by Nurse Drawing
Blood Sample

KOURI - M.A.

1003536230

HUMAN AHH STUDIES

Development of Rapid Assay Procedure

and

Collaborative Studies with University of Southern California

CTR Contract #
(Supplementary funding as 2FS)
MA Contract # 2225
(Supplementary funding as 2220)

CONTRACT PROPOSAL

for the period

Nov 1, 1974 - Dec 31, 1975

Vincent L. Ruwet

Vincent L. Ruwet
Vice-President, Contracts
and Administration

Sept 5, 1974

1003536231

TO: Council for Tobacco Research
110 East 59th Street
New York, New York 10022

FROM: Microbiological Associates, A Division
of Dynasciences Corporation
4733 Bethesda Avenue
Bethesda, Maryland 20014

DATE: September 3, 1974

Prepared by

Richard E. Kouri, Ph.D.
Project Director

1003536232

TABLE OF CONTENTS

1. Introduction

The Council for Tobacco Research, is engaged in a program to reduce carcinogenic and other hazards of smoking to human health. To this end, the determination of relative risk of particular populations to smoke-associated neoplasias is one such goal. This proposal outlines specific approaches that should provide a major step in achieving this goal. At the end of the proposed 14 months of experimentation, one or more standard assay procedure for the determination of levels of aryl hydrocarbon hydroxylase (AHH) in human populations will be available. Using such assay procedures, the relationship between levels of AHH inducibility and at least eight different cancers (including cigarette-smoke-associated lung carcinomas) will be established.

II. Background.

Aryl hydrocarbon hydroxylase (AHH) is the name given to one of the multi-component microsomal-bound enzymes (1,2). The actual steps involved in this oxidative metabolism are unknown; however, the best guess is that the substrate combines with the oxidized form of a carbon monoxide sensitive hemoprotein called cytochrome P-450. The substrate-cytochrome P-450 complex is then reduced by an electron donated by NADPH-cytochrome c reductase, to form a reduced substrate-cytochrome P-450 complex. This complex in turn reacts with molecular oxygen to form a reduced substrate-cytochrome P-450-O_2 complex. A second electron is then added to this complex to yield an active oxygen intermediate which decomposes with the formation of the product and the oxidized P-450. The product of this reaction, if polycyclic aromatic hydrocarbons (PAH) are substrates, are probably epoxides (3,4). These epoxide intermediates then:

1. Rearrange spontaneously to form phenols,
2. Are enzymatically metabolized to the dihydrodiol via the enzyme epoxide hydrase, or
3. Are enzymatically conjugated with glutathione using the enzyme glutathione conjugase.

These enzymes have two properties which make them uniquely important to the study of chemical carcinogenesis. First, metabolism of many substrates (especially PAH) does not necessarily result in detoxification, but rather are converted to water-soluble forms via transient chemically-reactive intermediates that are both cytotoxic (5,7) and carcinogenic (8,9). Second, this enzyme system is inducible by certain substrates, and this induction results in the enhanced metabolism of many foreign compounds (10). This latter property is important because, if these metabolic pathways for PAH are etiologically important in the initiation of chemical carcinogenesis, any marked changes in rates of formation of the water-soluble metabolites (e.g., epoxides, phenols, dihydrodiols and glutathione conjugates) or covalently bound metabolites, should affect the host's susceptibility to cancer.

In the house mouse, Mus musculus, not only is AHH in-

duced by treatment with certain substrates, but this inducibility also is host gene regulated. Treatment with phenobarbitol (PB) increases the metabolism of most of the drug substrates in every strain of mouse tested (11). Treatment with 3-methylcholanthrene (MCA) increases the metabolism of very few substrates and in only particular strains (12). These differences probably result from the fact that PB causes a rapid nonspecific proliferation of constitutive AHH (13), while MCA induces a new spectrally distinct cytochrome called P^1-450 or P448 (14,15) which has different substrate specificities. The ability to respond to MCA (but not PB) segregates as a single autosomal gene in crosses involving the C57BL/6 (B6) and DBA/2 (D2) strains of mice (12, 16, 17, 18). We proposed this locus be designated Ah; the allele carried by the B6 mouse (inducible) is Ah^b, and the allele carried by the D2 mouse (noninducible) is Ah^d. Following treatment with MCA, the differences between the AHH levels in various tissues of Ah^b/Ah^b or Ah^d/Ah^d mice are 2-80 fold greater than that of Ah^d/Ah^d animals (16,19). Thus, one can evaluate tumor susceptibility among litter-mates in which the presence or absence of AHH induction is expressed in their tissues. With the use of such a model, other nonspecific strain differences - such as characteristic mouse strain differences involving immunology, latent viral infections, nutrition, hormones, stress, or levels of other enzymes - will be theoretically cancelled.

Using this model system, we have reported that segregants carrying the Ah^b allele are approximately 12 times more sensitive to MCA induced fibrosarcomas than animals homozygous for the Ah^d allele (20,21). Thus, it seems likely that the types of metabolites, or just the quantity of these metabolites determined by this novel "inducible" enzyme play a major role in determining the susceptibility of mice to chemical carcinogenesis.

The human situation may be analogous to that described in mice. AHH induction is variable in humans (22); however, three distinct groups are observed: low, intermediate and high inducibility (22,23). Results suggest a single locus of genetic control, with gene frequency of the low and high alleles

being .717 and .283 respectively. To date, the information about phenotypic variations of AHH is limited to Caucasians. There is no information available, of which we are aware, indicating the frequency distribution of AHH among Blacks, Orientals American Indians, or specific ethnic populations. In this limited system, the only disease shown to harbor a possible association with AHH has been the published work by Kellerman, et al, demonstrating an association between AHH, cigarette smoking and bronchogenic carcinoma (24). This same group has data showing a similar association with carcinoma of the colon though this is unpublished, but was told to our group and presented in a very preliminary manner at the recent meetings of the American Society of Human Genetics at Atlanta, Georgia. This result suggests that PAH are important causes of cancer in humans and that, as in mice, those individuals with a heightened ability to metabolize PAH are more susceptible to the chemically-induced or "spontaneous" cancers.

These points are of extreme interest to cancer epidemiologists since it is well-known that cancer is not uniformly distributed in the population. Rather, there are marked variations in all specific anatomic varieties of cancer. Many of these variations correlate with different geographic areas, racial and ethnic groups and, in addition, familial factors, habit patterns, and occupational factors condition these variations significantly. In light of the limited knowledge available on AHH in differing populations in different clinical settings, as well as the limited knowledge with respect to disease associations, it would seem compelling that studies be developed to extend knowledge in these specific areas.

The major assays used to detect AHH activity both *in vitro* and *in vivo* have been modifications of the original one described by Nebert and Gelboin (25). The assay is based on the ability to spectrophotofluorometrically detect one of the phenolic metabolites of benzo(a)pyrene (BP), 3-hydroxybenzo(a)-pyrene (3-OH BP). The material being assayed is added to a small volume of buffer containing NADPH and Mg^{++}. The assay is initiated by the addition of BP and allowed to run at 37°C for 10 to 30 minutes. Cold acetone-hexane is added to stop

the reaction and to extract most of the metabolites as well as the unmetabolized BP. A sample of the hexane-acetone phase is extracted with 1N NaOH and the amount of 3-OH BP in the alkaline phase is determined. The assay is only limited to the resolving power of the fluorometric instrument; the limit being about 0.5 pmoles 3-OH BP per ml, or about 0.01 pmoles 3-OH BP per mg protein per minute. Advantages of this assay are that it is capable of monitoring both constitutive and induced levels of AHH, is highly sensitive and is relatively inexpensive. Disadvantages are the low levels of AHH which need to be determined in human studies are close to the sensitivity limits of this assay and extreme care must be taken to avoid wide day-to-day fluctuations. Factors such as pH of the buffer, temperature of the assay, purity of the solvents, or cleanliness of the glassware must be controlled. A modification (26) of this assay has been used to detect the levels in human lymphocytes. When care is taken, this assay can reproducibly detect those low levels of AHH, however, some of these modifications also possess inherent problems. For example, the lymphocytes must be activated by a mitogen and this activation step can be influenced by the physiological status of the donor and could be changed if the donor is on medications such as steroids, aspirin or immunosuppressants. This activation step is also very sensitive to changes in pH, temperature or types of growth medium (27). Another disadvantage is that the assay now takes 5 days to complete. This definitely limits the number of assays that can be performed. This assay can measure the AHH levels in human alveolar macrophages (28); however, the levels observed are even lower than those observed in cultured lymphocytes.

Three main problems must be worked out before this assay can be used in a large scale screening study of human population. First, a reproducible source of cells must be made available. Second, procedural modifications must be done so as to lower the rather high non-specific fluorescence observed in the zero-time control cultures. Third, the number of tests capable of being performed must be greatly increased.

Since previous studies have shown that the small lymphocyte is capable of giving reproducible AHH inducibility results (22, 26), this cell is probably the one of choice. The major ways to isolate lymphocytes are ammonium chloride-DEAE dextran precipitation and gradients, such as ficoll-hypaque. We feel the latter technique has certain advantages - mainly it yields a clean, relatively pure, "band" of small lymphocytes. In our laboratory, this "band" consists of cells in which greater than 85% are small lymphocytes. One way to express enzyme activity is units of activity per a certain number of cells (e.g. 10^6 cells). Thus, the more accurately one can determine the number of cells, the more reproducible the results. The isolated lymphocytes must be activated in vitro by mitogens, such as pokeweek mitogen (PW) or phytohemagglutanin (PHA). Doses of PW and PHA approximating 1% seem to give optimal activation (29). This activation step is very sensitive to changes in pH, temperature, or types of growth medium (27). To this end, large lots of medium (RPMI - #1640), fetal calf serum, mitogens, and antibiotics must be purchased, pretested, and stored. Only then can a standard source of cells be relatively assured. The use of the small lymphocyte may have a secondary advantage because it is metabolically quiescent in vivo and is activated in vitro. Many in vivo physiological changes, such as drug treatment, diseases, exposures to pollutants, etc., may only negligibly affect the lymphocyte.

For the standardization of the assay, the optimal pH, temperature, incubation time, cell concentration, and buffer will be determined. The effect of solvent and/or various inducers will be tested. The chemical, 7,8-benzoflavone will probably be the inducer of choice because it can maximally induce AHH and is not carcinogemic.

The assay itself has inherent problems because of two major facts:

a. The level of enzymes observed in the cultured lymphocytes, although discernible, approaches the sensitivity limits of this assay, and

b. Levels of AHH in the three human subpopulations are

not that different.

The latter problem is exemplified by the fact that inducibility levels (relative increases over vehicle-treated control levels) of 2, 3, and 4 represent the ideal low, intermediate and high AHH inducibility subgroups of the human population. These two problems can be largely rectified if one concentrates on one particular facet of the assay - the relative difference between the non-induced (constitutive) AHH activity and zero time control values. The constitutive activity is that level of enzyme observed in mitogen-activated, vehicle-treated, control cultures. The zero time control contains the complete reaction mixture plus cells and BP, but in which cold acetone-hexane was added before incubation. If the constitutive activity is not sufficiently greater than this zero time control, then small deviations in the zero time activity will greatly affect the AHH inducibility ratios. For example, typical results may be:

AHH (Fluorescent units)

Constitutive	Induced
0.8	1.6

If zero time is 0.3 then inducibility is $\frac{1.6-0.3}{0.8-0.3}$ or 2.6

If zero time is 0.1 then inducibility is $\frac{1.6-0.1}{0.8-0.1}$ or 2.1

Therefore, this individual could be either a low inducer or an intermediate inducer, depending on which zero time was observed. The major ways to increase the difference between the zero time and constitutive fluorescence values are:

a. The use of more cells;

b. Use of longer incubation periods (the assay is linear for up to 1 hour);

c. Use of carefully cleaned glassware (soap with no brighteners), and

d. Use of highly purified reagents.

For the standard assay, negligible zero time fluorescence relative to constitutive activity (<10%) will be the goal.

III. Experimental

A. Use of fresh blood.

Recent information from our laboratories has demonstrated that blood can be kept at room temperature (or shipped at room temperature) for at least 24 hours and the lymphocytes can still be activated and assayed. The following protocol has been tentatively established:

1. Collect 20 ml venous blood (in heparinized tubes) between 11:00 AM and 1:00 PM (PCT);
2. Send heparinized tubes of whole blood via regularly scheduled airlines from L.A. International Airport to Dulles International Airport. American Airlines Flight #110 at 2:30 PM is probably the best. Samples arrive at 11:30 PM (EST) and are kept at room temperature until the next morning for pick up at the Airport at 8:00 AM and brought to the laboratory;
3. At that time, dilute blood 20% with medium;
4. Layer 9 ml aliquots of diluted blood onto 6 ml ficoll-hypaque solution;
5. Centrifuge 45 minutes, 1200 xg;
6. Collect lymphocyte "band" and wash twice with medium;
7. Count cells in an automatic counter, adjust to 0.5 X 10^6/ml, and add 1.0 ml per incubation tube;
8. Medium will be RPMI #1640 supplemented with 20% fetal calf serum, antibiotics, 1% PHA and 1% PM;
9. Incubate 72 hours at 37°C, 5% CO_2;
10. Add 0.01 ml of 0.75mM 3-methylcholanthrene or 7,8-benzoflavone to certain cultures and 0.01 ml acetone to controls;
11. Twenty-four hours later, count cells, pellet at 1,000 xg for 10 minutes and resuspend cells in a 0.1M tris-chloride buffer (pH 8.5) containing 2.7 mg bovine serum albumin, 0.8 μg NADPH, and 0.006M $MgCl_2$ at a concentration of 4×10^6 cells per ml.
12. Add 80nMoles BP in a total of 0.010ml acetone to each tube and incubate for 45 min at 37°C in air with shaking.

13. Stop the assay with cold acetone-hexane (1:3);
14. After shaking the two phases of 37°C for an additional 10 min, extract a 3.0 ml aliquot of the upper hexane phase with 1.0 ml in NaOH;
15. Quantitate the amount of 3-OH BP in the alkaline phase in an Aminco-Bowman spectrophotofluorometer with excitation at 396nm and emission at 522nm;
16. Express data in pMoles 3-OH BP formed per min per 10^6 cells.

These procedural changes definitely limit the number of assays that can be performed. One technician can only handle about 20-30 individual patients per week.

B. Use of frozen samples.

The interposition of a freezing step in this aforementioned protocol should have the following advantages: (1) transfer and delegation of some of the early procedural steps to the "collective" laboratories, thus allowing for an increased number of assays to be performed, and (2) circumventing a possible major problem when using fresh material; that is, rapid, efficient and responsible shipping.

1. Use of frozen lymphocytes.

A freezing step can be introduced after lymphocyte isolation. The cells must be slow-frozen according to standard procedures and held at -120°C. The cells can then be shipped in dry ice containers and held until 20-30 samples have been accumulated. At this time, the cells can be thawed, cultured, activated, and assayed. The use of this freezing step should double or triple the number of assays that can be performed per week.

2. Use of frozen-activated-induced cells.

A freezing step can also be worked in after the cells have been collected, activated, and induced. The induced cells will be collected as described in steps 4-16 of the aforementioned protocol, however, the induced and control cells will be stored at -70°C in a pellet form with 0.2ml tris-HCl buffer on top of this pellet. When 100-300 frozen samples have been

collected, all can be assayed on the same day. In this way, one central laboratory can run the assay for various "collection" centers. Large pools of lymphocytes derived from known low, intermediate and high AHH inducers could be frozen and run concomitantly with test cells. This could serve as an invaluable internal assay control. This procedural alteration could increase the number of assays performed probably by 5 to 10 fold.

C. Source of cells.

The population of Menck et al (30) appears to fulfill the criteria of accessibility as well as availability of adequate numbers of patients. The initial study will be concentrated on the lung cancer population. 50-100 lung cancer patients, 50-100 hospital controls and 50-100 non-hospitalized controls will be assayed. Assays will be done at MA at a rate of 20-50 per week. A confirmatory assay under the supervision of Drs. J. Brown and R. Gordon (USC) will also be done, but only at a rather low level (about 4-10 per week). This study will also entail a limited questionnaire involvement; including history of cigarette smoking, drug exposure, occupation etc. Medical verification of the pathological lesion will also be done.

Beginning Oct-Nov, 1974, the suggested approach will concentrate on the importance of cancer association of AHH inducibility. A matched population would seem to be the best obtainable control at this level. The requirement for detailed questionnaire and multi-variant analysis of same must be incorporated to observe correlates and associations. Smoking habits, while normally considered a tertiary study variable, are included in this secondary study, since this variable has particular interest to the granting agency. The procedure for this secondary study will include one hundred patients for each of seven cancer types (total 700) and 700 to 800 controls matched for age, sex, ethnic group and smoking habits. The routine fluorometric assay discussed before will be done. However, concurrent preparations for automated analysis will be included in preparation for the tertiary level studies. The questionnaire for this study will be detailed and organized for computeriza-

tion. This indepth level of inquiry would give operational experience and possible leads for specific indepth tertiary studies. This detailed questionnaire is compatible with the 1,500 patients projected. Areas to be emphasized in this secondary level include: 1) normal medical history; 2) detailed employment history - with master breakdown code of probable PAH, etc. exposure; 3) detailed drug history, weighted for those drugs involved in AHH; 4) detailed environmental history and breakdown code; 5) detailed smoking history; 6) detailed alcoholic consumption history; 7) other variables to be defined - possible psychological evaluations.

The questionnaire validity will be important. Analysis should be undertaken for possible correlations as the data accumulates. These correlations can be used to predict, direct, and avoid unnecessary duplication in the following studies.

IV. References

1. Mason, H.S. Mechanisms of oxygen metabolism. Adv. Enzymol. 19: 79-233, 1957.

2. Conney, A.H. Pharmacological implications of microsomal enzyme induction. Pharmacol., Rev., 19: 307-366, 1967.

3. Grover, P.L., Hewer, A., and Sims, P. Epoxides as microsomalmetabolites of polycyclic hydrocarbons. FEBS Letters, 18: 76-80, 1971.

4. Selkirk, J.K., Huberman, E., and Heidelberger, C. An epoxide is an intermediate in the microsomal metabolism of the chemical carcinogen, dibenz(a, h)anthracene. Biochem. Biophys. Res. Commun., 43: 1010-1016, 1971.

5. Gelboin, H.V., Huberman, E., and Sachs, L. Enzymatic hydroxylation of benzo(a)pyrene and its relationship to cytotoxicity. Proc. Natl. Sci., U.S.A., 65: 1188-1194, 1969.

6. Brown, D.O., Lubet, R.A., and Kouri, R.E. The relationship of aryl hydrocarbon hydroxylase (AHH) to benzo(a)pyrene-induced cytotoxicity in cell cultures of hamster fetuses. Proc. Am. Assoc. Cancer Res., 12: 50, 1971.

7. Lubet, R.A., Brown, D.O., and Kouri, R.E. The role 3-OH benzo(a)pyrene in mediating benzo(a)pyrene induced toxicity and transformation in cell culture. Res. Comm. in Chem. Path. and Pharm. 6: 929-942, 1973.

8. Gelboin, H.V., Weibel, F.W., and Diamond, L. Dimethylbenzanthracene tumorigenesis and aryl hydrocarbon hydroxylase in mouse skin: Inhibition of 7,8-benzoflavone. Science, 170: 169-170, 1970.

9. Marquardt, H., Kuroki, T., Humberman, E., et al. Malignant transformation of cells derived from mouse prostate by epoxides

and other derivatives of polycyclic hydrocarbons. Cancer Res., 32: 716-720, 1972.

10. Conney, A.H., and Burns, J.J. Metabolic interactions among environmental chemicals and drugs. Science 178: 576-586, 1972.

11. Gielen, J.E., and Nebert, D.W. Microsomal hydroxylase induction in liver cell culture by phenobarbital, polycyclic hydrocarbons, and p'p'-DDT. Science 172: 167-169, 1971.

12. Thomas, P.E., Kouri, R.E., Hutton, J.J. The genetics of aryl hydrocarbons hydroxylase induction in mice: A single gene difference between C57BL/6 and DBA/2J. Biochem. Genet. 6: 157-168, 1972.

13. Gielen, J.E., and Nebert, D. W. Aryl hydrocarbon hydroxylase induction in mammalian liver cell culture. 1. Stimulation of enzyme activity in nonhepatic cells and in hepatic cells by phenobarbital, polycyclic hydrocarbons and 2,2-bis(p-chlorophenyl)-1, 1, 1-trichloroethane. J. Biol. Chem., 246: 5189-5198, 1971.

14. Sladek, N.E., and Mannering, G.J. Evidence for a new P-450 hemoprotein in hepatic microsomes from methylcholanthrene treated rats. Biochem. Biophys. Res. Commun., 30: 607-612, 1966.

15. Alvares, A. P., Schilling, G., Levin, W., and Kuntzman, R. Studies on the induction of CO-binding pigments in liver microsomes by phenobarbital and 3-methylcholanthrene. Biochem. Biophys. Res. Commun., 29: 521-526, 1967.

16. Nebert, D. W., Goujon, F., and Gielen, J. E. Aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons: simple autosomal dominant trait in the mouse. Nature, New Biol., 236: 107-110, 1972.

17. Gielen, J.E., Goujon, F.M., and Nebert, D. W. Genetic

regulation of aryl hydrocarbon hydroxylase induction. II. Simple mendelian expression in mouse tissues in vivo. J. Biol. Chem., 247: 1125-1137, 1972.

18. Nebert, D. W., Benedict, W. F., and Kouri, R.E. Aromatic hydrocarbon-produced tumorigenesis and the genetic difference in aryl hydrocarbon hydroxylase induction. In World Symposium on Model Systems in Chemical Carcinogenesis. (DiPaolo, J., Ts'o, P., eds.) Cleveland, Ohio, Chemical Rubber Co., 1973. (In Press)

19. Kouri, R.E., Salerno, R. A., and Whitmire, C. E. Relationship between aryl hydrocarbon hydroxylase inducibility and sensitivity to chemically induced subcutaneous sarcomas in various strains of mice. J. Natl. Cancer Inst., 50: 363-368, 1973.

20. Kouri, R. E., Ratrie, H., and Whitmire, C. E. Evidence of a genetic relationship between subcutaneous tumors and methylcholanthrene-induced subcutaneous tumors and inducibility of aryl hydrocarbon hydroxylase. J. Natl. Cancer Inst. 51: 197-200, 1973.

21. Kouri, R. E., Ratrie, H., and Whitmire, C. E. Genetic control of susceptibility of 3-methylcholanthrene-induced subcutaneous sarcomas. Int. J. Cancer 13: 714-720, 1974.

22. Kellermann, G., Cantrell, E., and Shaw, C. Variations in extent of aryl hydrocarbon hydroxylase induction in cultured human lymphocytes. Cancer Res., 33: 1654-1656, 1973.

23. Kellermann, G., Luyten-Kellermann, M., and Shaw, C.R. Genetic variation in human lymphocytes. Amer. J. Human Genetics 25: 327-331, 1974.

24. Kellermann, G., Shaw, C.R., and Luyten-Kellermann, M. Aryl hydrocarbon hydroxylase inducibility and bronchogenic carcinoma. New England J. Med., 289: 934-936, 1973.

25. Nebert, D. W., and Gelboin, H.V. Substate-inducible microsomal aryl hydrocarbon hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. J. Biol. Chem., 268-249, 1968.

26. Busbee, D. L., Shaw, C.R., and Cantrell, E. T. Aryl hydrocarbon hydroxylase induction in human leukocytes. Science 178: 315-316, 1972.

27. Johnson, L, I., and Rubin, A.D. Lymphocyte growth and proliferation in culture. 1970. (A. S. Gordon, ed.) Regulation of Hematopoiesis, Vol. II, white cell and platelet production. Appleton-Century-Crofts, New York, New York, pg. 1477-1525.

28. Cantrell, E. T., Warr, G. A., Busbee, D. L., and Martin, R. R. Induction of aryl hydrocarbon hydroxylase in human pulmonary alveolar macrophages by cigarette smoking. J. Clin. Invest. 52: 1881-1884, 1973.

29. Cantrell, E., and Busbee, D. Effects of mitogens and methylcholanthrene on aryl hydrocarbon hydroxylase in cultured human leukocytes. Mol. Pharmacol. (In Press) 1974

30. Menck, H. R., Casagrande, J. T., Henderson, B. E. Industrial air pollution: possible effect on lung cancer. Science, 183: 210-212, 1974.

Soc. Sec. No. 273-38-2503

V. CURRICULUM VITAE - RICHARD E. KOURI

BIRTH: REDACTED

EDUCATION:	1970	Ph. D.,	Radiation Biology University of Tennessee, Knoxville
	1968	M. S.,	Radiation Virology University of Tennessee, Knoxville
	1965	B. S.,	Microbiology Ohio State University, Columbus

PROFESSIONAL AFFILIATIONS:

REDACTED

REDACTED

PRESENT POSITION: 1974 - present

REDACTED

REDACTED

POSITION DESCRIPTION: Development and application of in vitro and in vivo viral-chemical carcinogenesis assay systems for tobacco smoke and smoke components, and the role of viruses and chemicals in the etiology of cancer. The role of the enzyme complex, aryl hydrocarbon hydroxylase, in cancers of both animals and man.

1003536250

PRIOR
EXPERIENCE: 1972 - 1974

REDACTED

1971 - 1972

REDACTED

1970 - 1971

REDACTED

REDACTED

1967 - 1970

1966 - 1970

1966 - 1967

REDACTED

PUBLICATIONS - RICHARD E. KOURI

Kouri, R. E., and Coggin, J. H. Radiation Responses of Embryonal and SV40 Transformed Hamster Cells in Culture. Proc. Soc. Exp. Biol. Med., 129: 609-620, 1968.

Kouri, R. E., Lubet, R. A., and Brown, D. Q. In Vitro Cellular Response to Benzo(a)pyrene Measured by a Microfluorometric Technique. J. Cell Biology, 43: 72a, 1969.

Kouri, R. E., Lubet, R. A., and Brown, D. Q. Effects of X-rays on Uptake of a Chemical Carcinogen, Benzo(a)pyrene, in Individual Cells in Culture. Rad. Res., 43: (Gb-5) 262-263, 1970.

Brown, D. Q., Lubet, R. A., and Kouri, R. E. The Relationship of Aryl Hydrocarbon Hydroxylase (AHH) to Benzo(a)pyrene (BP) Induced Cytotoxicity in Cell Cultures of Hamster Fetuses. Proc. Am. Assoc. Cancer Res., 12(197): 50, 1971.

Kouri, R. E., Lubet, R. A., and Brown, D. Q. In Vitro Cellular Uptake of Benzo(a)pyrene Measured by a Microfluorometric Technique. Proc. Soc. Exp. Biol. Med., 136: 1038-1044, 1971.

Miller, O. J., Miller, D. A., Kouri, R. E., Allerdice, P. W., Dev, V. G., Grewal, M. S., and Hutton, J. J. Identification of the Mouse Karotype by Quinacrine Fluorescence and Tentative Assignment of Seven Linkage Groups. Proc. Nat. Acad. Sci., 68: 1530-1533, 1971.

Kouri, R. E., Lubet, R. A., and Brown, D. Q. X-Irradiation Enhanced Benzo(a)pyrene Carcinogenesis In Vitro. Rad. Res., 47: (Gb-9), 1971.

Kouri, R. E., Miller, D. A., Miller, O. J., Dev, V. G., Grewal, M. S., and Hutton, J. J. Identification by Quinacrine Fluorescence of the Chromosome Carrying Mouse Linkage Group 1 in the Cattanach Translocation. Genetics, 69: 129-132, 1971.

Miller, D. A., Kouri, R. E., Dev, V. G., Grewal, M. S., Hutton, J. J., and Miller, O. J. Assignment of Four Linkage Groups to Chromosome in Mus Musculus and a Cytogenetic Method for Locating Their Centromeric Ends. Proc. Nat. Acad. Sci., 68: 2699-2702, 1971.

Dev, V. G., Grewal, M. S., Miller, D. A., Kouri, R. E., Hutton, J. J., and Miller, O. J. The Quinacrine Fluorescence Karyotype of Mus Musculus and Demonstration of Strain Differences in Secondary Constrictions. Cytogenetics, 10: 436-451, 1971.

Miller, O. J., Miller, D. A., Kouri, R. E., Dev, V. G., Grewal, M. S., and Hutton, J. J. Assignment of Linkage Groups VIII and X to Chromosomes in Mus Musculus and Identification of the Centromeric End of Linkage Group 1. Cytogenetics, 10: 452-464, 1971.

Benedict, W. F., and Kouri, R. E. Ara-C-Produced Transformation in Hamster Fetal Cells. Proc. Amer. Assoc. Cancer Res., 14: 94, 1972.

Kouri, R. E., Lubet, R. A., and Brown, D. Q. Quantitation of Aryl Hydrocarbon Hydroxylase Activity in Individual Hamster Fetal Cells In Vitro. J. Nat. Cancer Inst., 49: 993-1005, 1972.

Thomas, P. E., Kouri, R. E., and Hutton, J. J. The Genetics of Aryl Hydrocarbon Hydroxylase Induction in Mice: A Single Gene Difference Between C57BL/6 and DBA/2J. Biochemical Genetics, 6: 157-168, 1973.

Miller, D. A., Allerdice, P. W., Kouri, R. E., Dev, V. G., Grewal, M. S., Miller, P. J., and Hutton, J. J. Quinacrine Fluorescent Chromosome Analysis of the Snell Translocation in the Mouse. Genetics, 17: 633-639, 1972.

Benedict, W. F., and Kouri, R. E. The Relationship Between 1-B-D-arabinofuranosylcytosine (Ara-C) Transformation and Chromosomal Changes in Hamster Fetal Cells. Genetics, 74: 195-205, 1973.

Benedict, W. F., Karon, M., and Kouri, R. E. Malignant Transformation Produced by Cytosine Arabinoside. Pediatric Res. (In Press, 1973)

Kouri, R. E., Salerno, R. A., and Whitmire, C. E. Relationships Between Aryl Hydrocarbon Hydroxylase Inducibility and Sensitivity to Chemically-Induced Subcutaneous Sarcomas in Various Strains of Mice. J. Nat. Cancer Inst., 50: 363-368, 1973.

Nebert, D. W., Benedict, W. F., and Kouri, R. E. Aromatic Hydrocarbon Produced Tumorigenesis and the Genetic Differences in Aryl Hydrocarbon Hydroxylase Induction. In: P. Ts'o and J. DiPaolo (eds). World Symposium on Model Studies in Chemical Carcinogenesis, Chemical Rubber Co., Cleveland, Ohio. (In Press, 1973)

Kouri, R. E., Ratrie, H., and Whitmire, C. E. Evidence for Genetic Relationship Between Susceptibility to 3-Methylcholanthrene Induced Subcutaneous Tumors and Inducibility of Aryl-Hydrocarbon Hydroxylase. J. Nat. Cancer Inst., 51: 197-200, 1973.

Lubet, R. A., Brown, D. Q., and Kouri, R. E. The Role of 3-OH-Benzo(a)pyrene in Mediating Benzo(a)pyrene Induced Cytotoxicity and Transformation in Cell Culture. Res. Comm. in Chem. Path. and Pharm., 6: 929-942, 1973.

Kouri, R. E., Ratrie, H., and Whitmire, C. E. Genetic Control of Susceptibility to 3-Methylcholanthrene-Induced Subcutaneous Sarcomas. Int. J. Cancer, 13: 714-720, 1974.

Zimmerman, E. M., Kouri, R. E., Higuchi, K., Laird, F., and Freeman, A. E. Uptake, Metabolism and Persistence of 3-Methylcholanthrene in Rat Embryo Cells Infected with Murine Leukemia Virus. Cancer Res. (In Press, 1974)

Kouri, R. E., Kiefer, R., and Zimmerman, E. M. Hydrocarbon Metabolizing Activity of Various Mammalian Cells in Culture. In Vitro. (In Press, July - August, 1974)

Kouri, R. E., Ratrie, H., Atlas, S., Nina, A., and Nebert, D. W., Aryl Hydrocarbon Hydroxylase Induction in Human Lymphocyte Cultures by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Life Sciences. (In Press, 1974)

Kouri, R. E., Demoise, C. F., and Whitmire, C. E. The Significance of the Aryl Hydrocarbon Hydroxylase Enzyme Systems in the Selection of Model Systems for Respiratory Carcinogenesis. In: E. Karbe and J. F. Park (eds). Experimental Respiratory Carcinogenesis and Bioassays. (In Press, 1974)

Demoise, C. F., Kouri, R. E., and Whitmire, C. E. Cell-Mediated Immunity After Intratracheal Exposure to 3-Methylcholanthrene and Its Relationship to Tumor Transplant Growth in C3H/f Mai Mice. In: E. Karbe and J. F. Park (eds). Experimental Respiratory Carcinogenesis and Bioassays. (In Press, 1974)

Whitmire, C. E., Demoise, C. F., and Kouri, R. E. The Role of the Host in the Development of In Vivo Models for Carcinogenesis Studies. In: E. Karbe and J. F. Park (eds). Experimental Respiratory Carcinogenesis and Bioassays. (In Press, 1974)

Benedict, W. F., Rucker, N., Mark, C., and Kouri, R. E. Correlation Between the Balance of Specific Chromosomes and the Expression of Malignancy in Hamster Cells. J. Nat. Cancer Inst. (In Press, 1974)

Kouri, R. E., Kurtz, S., Price, P., and Benedict, W. F. Studies on the Ara-C-Induced Transformation of Hamster and Rat Cells in Culture. (Submitted, 1974)

Kouri, R. E., Ratrie, H., and Whitmire, C. E. Genetic Control of Susceptibility to Cancers Induced by 3-Methylcholanthrene. Proc. XI International Cancer Congress. (In Press, 1974)

Schedule C

Equipment

1.	Compu -pet 100 (automatic pipetter) (Fisher Scientific)	$1,295.00
	- parts and accessories	315.00
2.	Autocytometer II (automatic cell counter) (Fisher Scientific)	4,500.00
3.	Automatic dilutor (Fisher Scientific)	400.00
	Total	$6,510.00

Schedule B. Other Direct Costs

Materials		
Media (MA)	$2,925.	
Blood Samples	585.	
Chemicals	5,148.	
Total		$8,658.
Expendable Supplies		
Quartz cuvettes	$1,170.	
Glassware (productions and reuseable)	2,340.	
Disposable plasticware	2,935.	
Total		$6,442.
Total Other Direct Costs		$15,100.

1003536257

Schedule A

Direct Labor Costs

Name & Position	Time on Project	Total hrs*	$/hr	Total $
R. E. Kouri, Ph.D. Project Director	10%	225	REDACTED	
Vacancy, Ph.D. Asst. Proj. Dir.	40%	899	REDACTED	
C. McKinney Technician	100%	2247	REDACTED	
Vacancy, Technician	100%	2247	REDACTED	
Total		5618		$26,967.
Total Direct Labor (+ 6% raise)				1,618.
TOTAL				$28,585.

*Based on 14 months (2427 hours or 2247 working hours) (Nov 1, 1974 - Dec 31, 1975).

VI BUDGET

(Based on 14 month period from Nov 1, 1974 to Dec 31, 1975)

A.	Total Direct Labor (Schedule A)	$ 28,585.00
B.	Overhead (115% of A)	32,872.00
C.	Other Direct Costs (Schedule B)	15,100.00
D.	Travel ($500.00/professional plus three trips L.A. - Wash. D.C.)	2,500.00
E.	Total (A-D)	$79,057.00
F.	G & A (16% of E)	12,649.00
G.	Total Costs	$91,706.00
H.	Fixed Fee (10%)	10,190.00
I.	Total Cost before equipment	$101,895.00
J.	Equipment (Schedule C)	6,510.00
K.	Total Cost	$108,406.00

INTERIM PROGRESS REPORT

for the period

July 1, 1974 to September 1, 1974

"Human AHH Studies"

CTR Contract # 24
MA Contract # 2225

Prepared by:

Richard E. Kouri, Ph.D.

Microbiological Associates
4733 Bethesda Avenue
Bethesda, Maryland 20014

1003536260

TO: Council for Tobacco Research
110 East 59th Street
New York, New York 10022

FROM: Microbiological Associates, A Division
of Dynasciences Corporation
4733 Bethesda Avenue
Bethesda, Maryland 20014

DATE: September 13, 1974

1003536261

Microbiological Associates
Interim Progress Report

CONTENTS

Microbiological Associates
Interim Progress Report

I. <u>Work Accomplished During the Report Period, Milestones</u>

A. Development of procedures to routinely and reproducibly isolate human lymphocytes.

During this report period, we have standardized our procedures for the isolation of lymphocytes from whole human blood. The procedure is as follows:

1. The area where blood is to be taken is washed with a sterile prepodyne swab (Clinipad Corporation, Stamford, Connecticut).

2. Venous blood is collected in sterile 150 ml evacuated containers (McGaw Laboratories, Milledgeville, Georgia) in which 1,000 units of sodium heparin have been previously added.

3. The blood is centrifuged at 500 rpm for 10 minutes in these same containers.

4. In 16 x 100 mm plastic tubes, 6 ml of a solution of ficoll:hypaque (s.g. 1.080) is added.

5. 9 ml aliquots of the clear plasma phase are added to the 6 ml gradients.

6. The lower phase consisting of the remaining lymphocytes, granulocytes and most of the red blood cells are diluted 20% with RPMI 1640 medium and 9 ml of the diluted blood is layered onto the gradients.

7. The ficoll-hypaque gradients are spun at 1500 rpm (590 xg) for 40 minutes.

8. The lymphocyte band (at the interface between the ficoll-hypaque and plasma) is collected with sterile Pasteur pipettes and transferred to 50 ml centrifuge tubes.

1003536263

9. Cells are washed twice with 25 ml aliquots of RPMI 1640 and cells are counted in an Autocytometer II (Fisher Scientific, Pittsburgh, Pennsylvania) and adjusted to a concentration of 0.5×10^6 cells/ml.

10. The complete medium is RPMI 1640, supplemented with 10% fetal calf serum, 50 units penicillin per ml, 50 μg streptomycin per ml, 1% phytohemaggutinin-M (Difco Laboratories, Detroit, Michigan), and 1% pokeweed mitogen (Grand Island Biological Company, Grand Island, New York).

11. One ml aliquots of cells are added to 10 x 75 mm plastic culture tubes and incubated at 37°, 5% CO_2 for 72 hours.

B. Determination of some of the parameters which influence the assay of aryl hydrocarbon hydroxylase using these human lymphocytes.

Tables 1 - 4 demonstrate some preliminary work on the effects of cell concentration, time of incubation, pH of incubation buffer, and effects of freezing on AHH levels of human lymphocytes. The source of enzyme was human lymphocytes cultured as described in the above Section A and treated with 7.5 nMoles MCA for 24 hours after the inital 72 hour activation period. The cells were pooled, collected by centrifugation, counted carefully in the Autocytometer II, and resuspended in a 0.1 M tris-HCl buffer, supplemented with 2.7 mg bovine serum albumin per ml, 0.8 μg NADPH per ml, 0.75 μg NADH per ml, and 0.006 M $MgCl_2$. The individual assay conditions are described in the footnotes to each table. Although preliminary in nature, the following conclusions seem apparent.

1. The assay is linear from cell concentrations of 1×10^6 to 8×10^6 cells per tube (Table 1), but the enzyme level, when 1×10^6 cells are present, is too close to zero time values to be reproducibly measured. 4×10^6 cells per tube seem to be the optimum concentration.

2. The assay is fairly linear with respect to time of incubation (Table 2). We are now routinely using a 45 minute incubation.

1003536264

3. The pH optimum seems to be much higher than the pH 7.5 - 7.8 normally adjusted for this assay (Busbee, et al, Science, 178, 315, 1972). Our optimum seems to be 8.5 - 8.7.

4. The assay is reproducible in at least two people's hands (Table 4) and frozen cultured-induced cells have AHH activity, but the inducibility (the relative increase of MCA treated cultures over untreated controls) is different from the fresh cultures. This may be a problem of handling the frozen material.

C. Effects of TCDD on AHH induction in human lymphocyte cultures.

Enclosed is a copy of a manuscript describing the effect of TCDD on the AHH activity of human lymphocyte cultures. Results indicate that:

1. TCDD can induce AHH at concentrations 40 to 60 times less than the concentration of MCA necessary for maximal hydroxylase induction;

2. The extent of induction by TCDD or MCA ranged between 1.7 and 2.9-fold for the 19 individuals assigned;

3. Those individuals with lower based and MCA-inducible hydroxylase activities in their lymphocytes also have lower TCDD-inducible hydroxylase activity, and

4. Although preliminary in nature, the observed variance of expression of hydroxylase induction more closely fits a unimodal, polygenic (i.e., more than 2 genes) pattern rather than a trimodal (single gene) form of inheritance.

II. Problems Encountered

A. Fluctuating zero times.

The zero time is the amount of non-specific fluorescence observed from samples in which the acetone-hexane phase has been added prior to incubation. This control contains all the ingredients of the assay, however, the assay is stopped before any enzyme activity accumulates. Results indicate that this value, which must be subtracted from the constitutive and induced enzyme levels and so very definitely influence the inducibility values of a given individual, is affected by such things as the purity of water, the kinds of tubes, the age of the BP-acetone substrate solution, and the purity of the organic solvents. We have tried to circumvent these problems by using 1) 4X-deionized distilled water from a Continental water system, 2) spectral grade acetone and hexane, 3) freshly made BP-acetone, using recrystallized BP, and 4) Kinble 15 x 125 mm sterile glass tubes. Hopefully, these conditions will give us a more reproducible zero time value.

B. Assay-to-assay variability.

The specific activity from one individual (i.e., amount of 3-OHBP formed per 10^6 cells per minute at 37°) either must be made more reproducible or the conditions that cause these variations must be recognized. For example, we may observe the following data one week:

Enzyme Source	Fluorescence Units
Zero time	0.15
Constitutive	1.2
MCA-induced	2.4

and the next week:

Enzyme Source	Fluorescence Units
Zero time	0.15
Constitutive	0.8
MCA-induced	1.2

In both cases, the enzyme level is low, and the individual is probably a low inducer, however, the specific activities are not near each other. This may be a problem with the activation step or the assay procedures; both steps must be studied. The use of one lot of reagents, such as medium, fetal calf serum, PHA, PWM, etc. as well as more experience with the logistics of the assay should help give more reproducible results.

C. Different lots of fetal calf serum.

Preliminary studies with three different lots of fetal calf serum indicate that this factor can have a tremendous effect on the growth and/or activation of the lymphocytes. We are currently reserving 6 lots of serum from Microbiological Associates and each will be tested for its ability to support the growth of lymphocytes. When a good lot is found, we will do all the assays for the entire length of this contract on this one lot.

D. The activation step.

The techniques of Yamamura (Clin. Exp. Immunol., 14: 457-467, 1973) seem to work very well for culturing human lymphocytes, however, the density needed to get good growth produces a major problem. We are currently culturing cells at a concentration of 0.5×10^6 cells/ml in a total of only one ml. Thus, for one individual who may yield 30×10^6 lymphocytes (≈ 30 ml whole blood), approximately 60 tubes must be cultured. This logistic problem is major if one tries to culture 50 to 10 people a day. The number of tubes is just too numerous to handle properly.

We intend to study this problem by using other culture vessels, such as petri dishes or erlenmeyer flasks, and use rocking platforms to keep the cells in suspension. Hopefully, one individual can be cultured in one vessel.

E. Effects of freezing.

In order to be able to do more assay per technician per day, we have initiated studies into the effects of freezing on this enzyme assay. The freezing step can be done at two places during the assay - slow freezing the isolated lymphocytes at -120^{o} and fast

freezing the cultured, activated, MCA-induced cells at -90°. Preliminary data indicate that AHH activity can be measured from cells frozen at either step, however, the values are at variance with those from fresh material. The problem with freezing whole cells probably centers on cell concentration, rate of freezing, rate of thawing, and perhaps time remaining frozen. The problems with freezing cultured, induced cells probably are the medium or buffer required for freezing, and the rates of freezing, and especially thawing, the material. These parameters will be tested in the next progress period.

II. Publication

Kouri, R. E., Ratrie, III, H., Atlas, S., Nina, A., and Nebert, D. W. Aryl Hydrocarbon Hydroxylase Induction in Human Lymphocyte Cultures by 2, 3, 7, 8-Tetrachloro-dibenzo-p-dioxin. Life Sciences, 1974, In Press.

TABLES

Microbiological Associates
Interim Progress Report

Table 1

Effect of Cell Concentration[a]

Source	1×10^6	2×10^6	4×10^6	8×10^6
HR[b] (ind.)	0.57 (1.6)[c]	1.1 (1.9)	2.2 (2.9)	4.5 (3.6)
(NI.)	0.35	0.58	0.75	1.25
CM[b] (ind.)	1.00 (2.5)	2.10 (3.0)	4.40 (3.4)	-
(NI.)	0.40	0.70	1.30	-

[a] Values given in terms of fluorescence units per tube. The assay was run at pH 8.5 for 45 minutes at 37°C.

[b] ind. = MCA treated cultures; NI. = non-induced controls

[c] The inducibility, the relative increase of AHH activity from MCA treated cultures over non-induced controls, is given parenthetically.

1003536271

Table 2

Effect of Time of Incubation[a, b]

Source	20'	40'	60'
CM (ind.)	1.50 (3.0)	3.20 (2.7)	4.80 (2.4)
(NI.)	0.50	1.20	2.00
MW (ind.)	1.40 (2.3)	2.20 (2.6)	4.00 (2.2)
(NI.)	0.60	0.85	1.80

[a] Values given in terms of fluorescence units per tube. The assay was run at pH 8.5 and each tube contained 4×10^6 lymphocytes.

[b] ind. = MCA treated cultures; NI. = non-induced controls

1003536272

-11-

Table 3

Effect of pH of Incubation Buffer[a, b]

Source	7.8	8.1	8.3	8.5	8.7	9.0
DA (ind.)	1.00 (3.3)	1.5 (3.0)	2.10 (3.8)	2.15 (3.6)	2.35 (2.9)	2.0 (2.7)
(NI.)	0.30	0.45	0.55	0.60	0.80	0.75
GG (ind.)	-	-	2.45 (3.7)	3.10 (3.9)	3.6 (3.6)	3.6 (4.0)
(NI.)	-	-	0.65	0.80	1.0	0.90

a Values given in terms of fluorescence units per tube. The assay was run for 45 minutes at 37°C and each tube contained 4×10^6 lymphocytes.

b ind. = MCA treated cultures; NI. = non-induced controls.

Table 4

Effect of Freezing (-70°) of Cultured - Induced Lymphocytes Prior to Assay and Effect of Two Different People Doing Assay on Same Day with Same Samples[a, b]

	Exp. I		Exp. II	
Source	Fresh	Frozen	Fresh	Frozen
GG (ind.)	4.4 (3.6)	3.55 (2.7)	4.20 (3.8)	3.70 (3.6)
(NI.)	1.15	1.26	1.15	1.10
RR (ind.)	1.15 (2.3)	1.35 (2.1)	1.15 (2.6)	1.30 (1.9)
(NI.)	0.50	0.65	0.45	0.70

[a] Values given in terms of fluorescence units per tube. The assay was run for 45 minutes at pH 8.5 and each tube contained 4×10^6 lymphocytes.

[b] Cultured, MCA-induced lymphocytes at 4×10^6 cells per tube were frozen as a pellet with 0.2 ml tris-HCl buffer (pH 8.3) atop this pellet.

1003536274

APPENDIX

(see attached paper)

ARYL HYDROCARBON HYDROXYLASE INDUCTION IN HUMAN LYMPHOCYTE CULTURES BY 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN

Richard E. Kouri, Harry Ratrie III,

Department of Viral-Chemical Oncology, Microbiological Associates, Inc. Bethesda, Maryland 20014

Steven A. Atlas, Akira Niwa, and Daniel W. Nebert

Section on Developmental Pharmacology
National Institute of Child Health and Human Development
National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT. Aryl hydrocarbon (benzo(a)pyrene) hydroxylase activity is induced in cultured human lymphocytes by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) at a concentration in the growth medium 40 to 60 times less than the concentration of 3-methylcholanthrene (MC) necessary for maximal hydroxylase induction. In cultured lymphocytes from 19 individuals, the extent of hydroxylase induction by TCDD or MC ranged between 1.7- and 2.9-fold. Those individuals having (presumably under genetic control) lower basal and MC-inducible hydroxylase activities in their lymphocytes also have lower TCDD-inducible hydroxylase activity. Although preliminary in nature, the data concerning the observed variance of expression of hydroxylase induction more closely fit a unimodal, polygenic (i.e. 2 or more genes) pattern rather than a trimodal (single gene) form of inheritance.

INTRODUCTION. The possible importance of aromatic hydroxylations of polycyclic hydrocarbons, drugs, and other environmental agents mediated by the membrane-bound monooxygenases to chemical carcinogenesis, pharmacology, and toxicology has been recently reviewed (1). Genetic differences in the induction of one such monooxygenase activity, the aryl hydrocarbon hydroxylase system, have been demonstrated in fetal mouse cell cultures (2), in mice (3-5), and in cultured human lymphocytes (6). An increased incidence of 3-methylcholanthrene-initiated sarcomas in mice (7-10) and, more recently, bronchiogenic carcinoma in man (11) has been highly correlated with the genetic "responsiveness" of the individual (i.e. the mouse or human having the hydroxylase activity most inducible by aromatic hydrocarbons).

Recent studies have shown (12) that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a toxic contaminant formed during the commercial

synthesis of the herbicide 2,4,5-trichlorophenoxyacetic acid, is approximately 30,000 times more potent than 3-methylcholanthrene (MC) as an inducer of aryl hydrocarbon hydroxylase activity in rat liver. Moreover, the hydroxylase activity in liver, kidney, bowel, lung, and skin of so-called "nonresponsive" mice is induced fully by TCDD, but not by MC or β-naphthoflavone (13,14). Because TCDD is metabolized so slowly in the rat, the biological half-life of this potent inducer is about 17 days (15) and the induced hydroxylase activity and associated cytochrome P_1450 remain elevated for more than 35 days (12). Thus, TCDD may become a serious environmental contaminant for man; evidence for the appearance of this toxic agent in the food chain has already been reported (16). Obvious questions arise. What dose of TCDD will be hazardous to man? What are the consequences of prolonged TCDD-induced aryl hydrocarbon hydroxylase activity in various human tissues? Do those individuals having genetically lower basal and MC-inducible hydroxylase activities also have lower TCDD-inducible hydroxylase activity in their lymphocytes? This last question is shown to be the case in this report.

EXPERIMENTAL PROCEDURE. Venous blood (usually 40cc) was collected in heparinized syringes from apparently healthy volunteers. No volunteer was currently on any medications. The whole blood was centrifuged at 200 x g for 15 min and 9 ml of the uppermost plasma-rich fraction was layered onto a 6ml ficoll-hypaque gradient (specific gravity 1.080) (17). At least 60% of the total lymphocyte yield - with the least number of contaminating red blood cells - exists in this plasma-rich fraction. In order to procure the remaining 40% of the lymphocytes, the remaining whole blood was diluted 20% with whole medium (RPMI #1640, 0.20 M HEPES buffer, 20% fetal calf serum and 50μg of gentamicin per ml (all products from Microbiological Associates, Inc., Bethesda, Maryland)) and similarly applied in 9ml aliquots to 6ml ficoll-hypaque gradients. Following a 1,000 x g centrifugation for 45 min, the lymphocyte "bands" were collected and combined, the lymphocytes were washed twice in whole medium, counted, and diluted to a concentration of about 0.8×10^6 cells per ml of whole medium. Five-ml cultures were made and bacto-phytohemagglutinin M (Difco Laboratories, Detroit, Michigan) and pokeweed mitogen (Grand Island Biological Co., Grand Island, New York) were added to final concentrations of 1% each, in order to "activate" the lymphocytes (increased metabolism, lymphoblast formation, and/or cell division usually occurs between one and three days in culture).

Stock solutions of 240μg of TCDD (kindly provided by Dr. A. Poland, University of Rochester School of Medicine and Dentistry, Rochester, New York) per ml of p-dioxane and 8.0mg of MC (Sigma Chemicals of St. Louis, Misouri) per ml of dimethylsulfoxide were diluted appropriately. The MC was purified by recrystallization from benzine before use. Dimethylsulfoxide and p-dioxane - at concentrations of 0.5% and 0.1%, respectively, or less - were not cytotoxic and did not affect the hydroxylase induction; the basal hydroxylase activity in this study was routinely determined in cultured lymphocytes exposed to 0.1% p-dioxane. Following incubation (37° with 5% CO_2) for 72 hours, the cells were treated with TCDD, MC, or p-dioxane alone in a volume of 0.01 ml. Twenty-four hours later, the cultures were agitated to break up clumps of cells, and the cells were counted in a Fisher autocytometer II cell counter (Fisher Instruments, Pittsburgh, Pennsylvania). The cells were then pelleted by centrifugation at 1,000 x g for 10 min and resuspended in 0.10 M Tris-chloride buffer, pH 7.8.

The enzyme assay and the Lowry protein determination were performed on the whole cells by means of published procedures (2, 3, 6). One unit of aryl hydrocarbon hydroxylase activity is defined (2-4) as that amount of enzyme catalyzing per min at 37° the formation of hydroxylated product causing fluorescence equivalent to that of 1 pMole of 3-hydroxybenzo(a)pyrene. Both duplicate and quadruplicate determinations were performed at different times, and the variability was almost always 10% or less. In this report, aryl hydrocarbon hydroxylase specific activity is expressed in either units per mg of cellular protein or units per 10^6 lymphocytes.

The data is given in terms of the inducible ratio (IR) which is the ratio of hydroxylase activity in TCDD- or MCA-treated lymphocytes to the enzyme activity in cultures treated with the solvent alone. The use of this parameter cancels out much of the normally occuring day-to-day variations associated with mitogen-activation, for regardless of the degree of activation, only the relative increase associated with TCDD or MCA treatment is being measured.

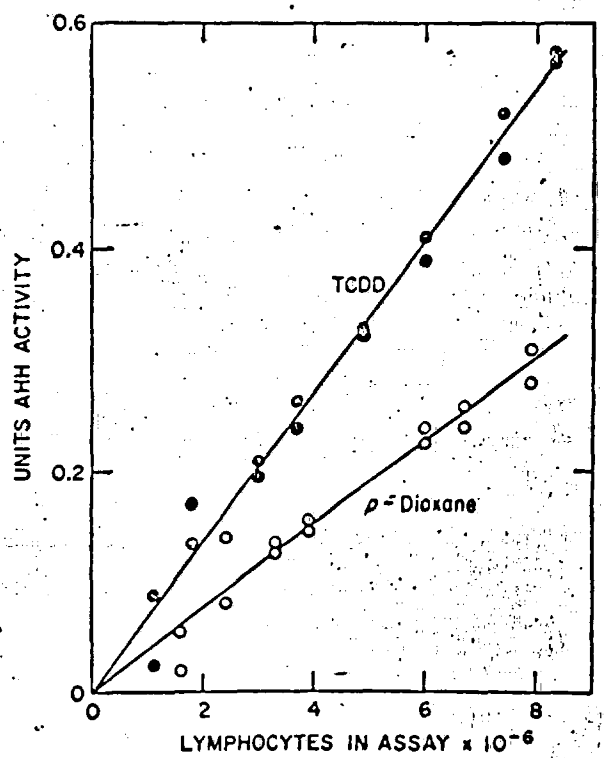
RESULTS AND DISCUSSION. Two major difficulties with the assay were encountered initially: a) large variations in the number of "mitogen-activated" lymphocytes at the end of 4 days in culture, and b) a high nonspecific fluorescence in the zero-time samples. The first problem was alleviated by increasing the purity of the lymphocyte preparations - by means of the ficoll-hypaque gradients as outlined above- thereby resulting in greater than 85% small lymphocytes. Since the enzyme activity appears to be associated with "mitogen-activated" lymphocytes, the highest yield of lymphocytes relative to other cell types should result in the most re-

1003536278

producible data. Also, since the data are expressed in terms of enzyme activity per given number of cells, the more reproducibly the number of lymphocytes can be quantitated, the more reproducible the data should be. The second problem was largely corrected by the use of more cells per assay tube, use of longer incubation periods (we have found the assay to be linear for at least 60 min) use of glassware cleaned carefully with soap containing no brighteners, and use of highly purified reagents. Although we found (Fig. 1) that the assay was linear at lymphocyte concentrations between 1×10^6 and 8×10^6 cells per assay tube, reproducibility was difficult with 3×10^6 cells or less. In fact, when 2×10^6 cells or less are assayed in the usual 1.0ml reaction mixture, we observe in the emission spectrum at about 522nm only a slight shoulder rather than a definitive peak. Currently we routinely use about 4×10^6 lymphocytes per assay tube and incubate

with

FIG. 1

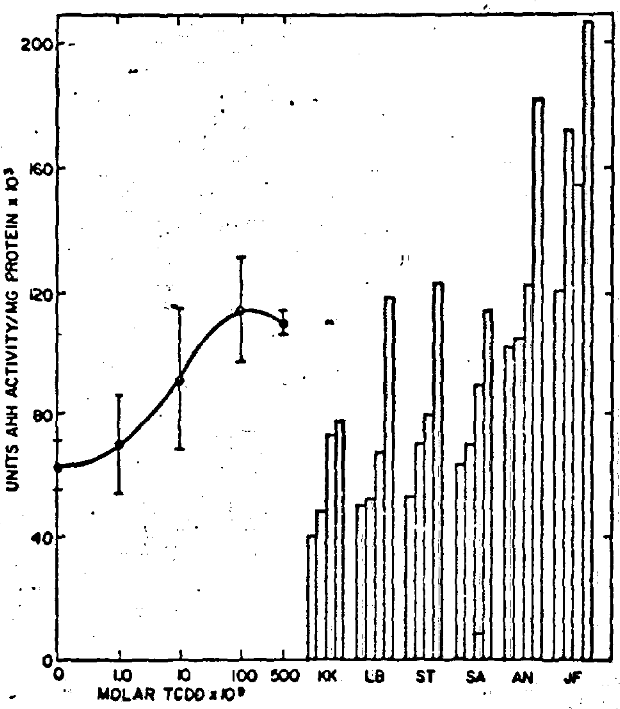


Aryl hydrocarbon hydroxylase (AHH activity as a function of number of lymphcytes used in the enzyme assay. The closed and open circles represent cells treated with 100nM TCDD and p-dioxane (0.1%), respectively, in the culture medium.

the substrate benzo[a]pyrene for 45 min. With the use of Kimble disposable 125 x 15 mm glass tubes (Kimble Glass Division, Owens-Illinois Glass Co., Toledo, Ohio) and spectral grade acetone and hexane (Fisher Scientific, Silver Spring, Maryland), fluorescence in the zero-time sample is now less than 10% of the fluorescence representing the basal enzyme activity. The zero-time sample contains the complete reaction mixture plus cells and benzo[a]pyrene, but to which cold acetone-hexane has been added prior to incubation. Similar values are obtained if the benzo[a]pyrene plus reaction mixture are incubated for the prescribed length of time and the cells are then added after the addition of cold acetone-hexane.

Fig. 2 shows the hydroxylase induction in response to varying concentra-

FIG. 2



Aryl hydrocarbon hydroxylase (AHH) induction by TCDD. At left is a dose-response curve representing 5 separate experiments on lymphocytes taken at different times from the same individual. The circles and brackets denote the mean ± S.E.M. Despite the large variations from one experiment to the next, the maximal extent of enzyme induction by TCDD in each experiment was reasonably constant (i.e. 1.4 to 1.7-fold) and approximated that by 1.5 μM MC.

At right are histograms representing six different individuals, whose initials appear below. The four bars, from left to right, depict the hydroxylase activity in lymphocytes treated with 0, 1.0, 10, and 100 nM TCDD, respectively. 100 nM represents about 33 ng per ml. Maximal induction in the six individuals, from left to right, is about 2.0, 2.4, 2.3, 1.8, 1.8, and 1.7, respectively.

tions of TCDD in the growth medium. Fifty per cent of the maximal induction (ED_{50}) was achieved with about 8 nM TCDD. The histograms in Fig. 2 and the data in Table I illustrate that, in lymphocytes from any given individual, the higher the basal hydroxylase activity, the higher the TCDD-inducible hydroxylase activity. Whether the hydroxylase specific activity is expressed in units per mg of cellular protein (Fig. 2) or in units per 10^6 cells (Table I), our conclusion is basically the same.

TABLE I

Effect of TCDD on aryl hydrocarbon hydroxylase induction in cultured human lymphocytes from four individuals

Dose of TCDD (nM)	Dose of TCDD (ng/ml)	R.K. SA[a]	R.K. IR[b]	D.A. SA	D.A. IR	G.G. SA	G.G. IR	M.W. SA	M.W. IR
0	0	0.039	(1.0)	0.042	(1.0)	0.050	(1.0)	0.050	(1.0)
0.3	0.10	0.043	1.1	0.046	1.1	0.055	1.1	0.050	1.0
3.0	1.0	0.055	1.4	0.067	1.6	0.100	2.0	0.065	1.3
30	10	0.077	2.0	0.088	2.1	0.130	2.6	0.140	2.8
300	100	0.079	2.0	0.094	2.2	0.141	2.8	0.145	2.9

[a]Specific activity (SA) is expressed as units of hydroxylase activity per 10^6 cells. These values represent the mean specific activity of 2 to 5 separate experiments performed at different times on lymphocytes from the same individuals.

[b]Inducibility ratio (IR) is the ratio of hydroxylase activity in TCDD-treated lymphocytes to the enzyme activity in cultures treated with the solvent p-dioxane alone.

Table II shows that the index of inducibility is about the same in lymphocytes from any given individual, when maximal inducing doses of either TCDD or MC are present in the culture medium. We estimate that the optimal inducing doses of TCDD and MC are about 30 nM and 1.5 μM, respectively; thus, TCDD is about 40 to 60 times more potent than MC as an inducer of hydroxylase activity in cultured human lymphocytes. This is in marked contrast to the 30,000-fold

TABLE II

Comparison of TCDD and MC as inducers of aryl hydrocarbon hydroxylase activity in cultured human lymphocytes from thirteen individuals

Volunteer's initials	Basal hydroxylase activity	TCDD (10 ng/ml or 30 nM)		MC (0.4 μg/ml or 1.5 μM)	
		SA[a]	IR[b]	SA	IR
P.G.	0.031	0.056	1.8	0.064	2.1
T.R.	0.033	0.059	1.8	0.064	1.9
K.T.	0.039	0.067	1.7	0.067	1.7
R.K.	0.039	0.078	2.0	0.070	1.8
A.L.	0.039	0.078	2.0	0.084	2.2
D.A.	0.042	0.088	2.1	0.084	2.0
S.G.	0.045	0.090	2.0	0.106	2.4
G.M.	0.048	0.098	2.0	0.092	1.9
H.R.	0.048	0.123	2.6	0.140	2.9
G.G.	0.050	0.132	2.6	0.126	2.5
M.W.	0.050	0.137	2.7	0.140	2.8
A.V.	0.053	0.140	2.6	0.137	2.6
C.M.	0.056	0.140	2.5	0.154	2.8

[a]Specific activity (SA) of the basal and induced enzyme is expressed as units of hydroxylase activity per 10^6 cells. These values represent the mean specific activity of at least two experiments performed at different times on lymphocytes from each individual.

[b]Inducibility ratio (IR) is the ratio of hydroxylase activity in TCDD- or MC-treated lymphocytes to the enzyme activity in cultures treated with p-dioxane alone. The correlation coefficients r for the relationship between the basal and induced hydroxylase activities from these 13 individuals are 0.82 and 0.73 for TCDD and MC, respectively ($P<0.01$ for both). This significant correlation between the basal and MC-induced enzyme activities is in agreement with the data of Kellermann et al. (6). However, our basal and inducible levels of hydroxylase activity in cultured lymphocytes are about 2 to 4 times lower than those reported by Kellermann and coworkers (6). We have since found that different levels of basal and inducible hydroxylase activities occur when lymphocytes from the same blood sample are grown in different lots of fetal calf serum, bacto-phytohemagglutinin M, and poke weed mitogen. The studies in this report were all performed with the same lots of these materials. For any large-scale comparison of genetic expression, therefore, the same lots of fetal calf serum, bacto-phytohemagglutinin M, and pokeweed mitogen should be constantly used throughout the entire study.

difference in potency between TCDD and MC in rat liver (12). This difference between 50-fold in culture and 30,000-fold in the intact animal is not understood and is under further investigation. It is possible that this effect reflects different binding affinities and/or tissue distribution differences between these two inducers in the intact animal that are not operant in cell culture. Hence, TCDD may be far more potent in man than what we observe in cultured human lymphocytes.

Several points concerning the assay of hydroxylase activity in lymphocytes should be emphasized. (i) The variance in the specific enzyme activity from leukocytes of the same individual from one week to the next is quite significant (e.g. the brackets in the dose-response curve of Fig. 2 represent the standard error of the mean). (ii) With either MC or TCDD as the inducer, we do not find "distinct classes of 'low,' 'intermediate,' and 'high' inducibility"--as was described by Kellermann and coworkers (6) with MC as the inducer. In fact, the greatest extent of hydroxylase induction we have yet found among 32 individuals (unpublished data) has been a factor of 2.9-fold. From the apparent Hardy-Weinberg distribution reported in the Houston population (6), we would have expected to see 2 or 3 individuals in the "high inducibility" group but we have found none in this "class." (iii) The enzyme induction even among various inbred strains of mice appears to involve at least 2, and probably more than 2, nonlinked genetic loci (5)--rather than the one locus as was first postulated (3, 4). These observations would lead us to believe that the observed variance of expression of hydroxylase induction in an outbred population--such as man--more closely fits a unimodal, polygenic, rather than a trimodal (single-gene), pattern of inheritance. This hypothesis is presently under further investigation.

1003536283

We have shown in this report a positive correlation between basal enzyme activity and the enzyme levels maximally inducible by either TCDD or MC. This threshold difference in response to aromatic hydrocarbon inducers has also been repeatedly observed with the various inbred strains of mice (2, 3, 13, 14). It is therefore possible that the more highly "responsive" individuals in the human population exposed to TCDD are more susceptible to any effects produced by prolonged elevated levels of induced hydroxylase activity. TCDD itself is not a potent carcinogen in mice; however, the synergistic action of TCDD with MC produces cancer in different strains of mice in direct proportion to the degree of elevation of the induced hydroxylase activity and associated cytochrome P_1450 content (R.E. Kouri, A.P. Poland, and D.W. Nebert, manuscript in preparation). The ~~facts~~ fact that TCDD induces aryl hydrocarbon hydroxylase activity in man and that this toxic compound is present in relatively high levels in certain parts of the world (16) suggest that, in addition to the short-term risk of TCDD because of toxic (18-24) and teratogenic (25, 26) properties, there may be considerable long-term risk because of possible synergism in chemically initiated tumorigenesis.

[This work was supported in part by contracts from the Council for Tobacco Research].

1003536284

REFERENCES

1. Daly, JW, Jerina, DM, & Witkop, B (1972) Experientia 28, 1129-1149
2. Nebert, DW, & Bausserman, LL (1970) J. Biol. Chem. 245, 6373-6382
3. Gielen, JE, Goujon, FM, & Nebert, DW (1972) J. Biol. Chem. 247, 1125-1137
4. Thomas, PE, Kouri, RE, & Hutton JJ (1972) Biochem. Genet. 6, 157-168
5. Robinson, JR, Considine, N, & Nebert, DW (1974) J. Biol. Chem. 249, in press
6. Kellermann, G, Luyten-Kellermann, M, & Shaw, CR (1973) Amer. J. Human Genet. 25, 327-331
7. Kouri, RE, Salerno, RA, & Whitmire, CE (1973) J. Nat. Cancer Inst. 50, 363-368
8. Kouri, RE, Ratrie, H, & Whitmire, CE (1973) J. Nat. Cancer Inst. 51, 197-200
9. Nebert, DW, Benedict, WF, & Kouri, RE (1974) In: Chemical Carcinogenesis, (POP Ts'o & JA Dipaolo, Eds.), (Marcel-Dekker, Inc.; N.Y., N.Y.), pp. 271-288
10. Kouri, RE, Ratrie III, H, & Whitmire, CE (1974) Int. J. Cancer 11, 714-720
11. Kellermann, G, Shaw, CR, & Luyten-Kellermann, M (1973) New Eng. J. Med. 289, 934-937
12. Poland, AP, & Glover, E (1974) Mol. Pharmacol. 10, 349-359
13. Nebert, DW, Robinson, JR, & Poland, AP (1973) Genetics 74, s193
14. Poland, AP, Glover, E, Robinson, JR, & Nebert, DW (1974) J. Biol. Chem. 249, in press
15. Piper, WN, Rose, JQ, & Gehring, PJ (1973) Adv. Chem. Ser. 120, 85-91
16. Baughman, R, & Meselson, M (1973) Environmental Health Perspectives, Experimental Issue No. 5, (NIEHS, Research Triangle, N.C.), 27-35
17. Böyum, A (1968) Scand. J. Clin. Lab. Invest. 176, 38-39
18. Schwetz, BA, Norris, JM, Sparschu, GL, Rowe, VK, Gehring, PJ, Emerson, JL, & Gerbig, CG (1973) Environmental Health Perspectives, Experimental Issue No. 5, (NIEHS: Research Triangle, N.C.), 87-99
19. Gupta, BN, Vos, JG, Moore, JA, Zinkl, JG, & Bullock, BC, ibid, 125-140
20. Vos, JG, Moore, JA, & Zinkl, JG, ibid, 149-162
21. Miller, RA, Norris, LA, & Hawkes, CL, ibid, 177-186
22. Lucier, GW, McDaniel, OS, Hook, GER, Fowler, B, Sonawane, BR, & Faeder, E, ibid, 199-209
23. Greig, JB, & De Matteis, F, ibid, 211-219
24. Poland, A, & Glover, E, ibid, 245-251
25. Neubert, D, Zens, P, Rothenwallner, A, & Merker, HJ, ibid, 67-79
26. Moore, JA, Gupta, BN, Zinkl, JG, & Vos, JG, ibid, 81-85

DOSIMETRY

1003536286

STOKELY-OAK RIDGE

1003536287

OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION
NUCLEAR DIVISION
POST OFFICE BOX X
OAK RIDGE, TENNESSEE 37830

September 16, 1974

To: Dr. John Kreisher, Council for Tobacco Research-USA

Re: Request for Supplemental Support

From: M. R. Guerin

As we have discussed, requests for special studies and special services to expedite your inhalation bioassay studies have been more numerous and have come sooner than was anticipated when our contract to evaluate exposure systems was negotiated. Examples of such "special studies/services", i.e., those not included in the negotiated contract, include:

(a) An immediate evaluation of the Process and Instruments (P & I) smoke exposure system.

(b) A pilot lung dosimetry experiment using mice exposed on the Walton-Horizontal exposure system.

(c) An evaluation of a novel animal containment system.

(d) A study of smoke uptake by tubings of varied construction.

(e) An in-depth operational evaluation of the LACS II and P & I systems to identify malfunctions. Neither system was operational on receipt.

We are presently discussing plans for a study of dosimetry attending the inhalation exposure of mice to smoke under various conditions. This study, to be carried out in collaboration with Microbiological Associates, is critical to identify conditions providing maximum exposure and to quantitate the exposure methodology.

1003536288

Re: Request for Supplemental Support
September 16, 1974
Page 2

Direct Support of other CTR-USA contractors is an important part of the CTR-USA/ORNL contract and should be continued. It is apparent, however, that these added responsibilities make it impossible to meet the commitments of the original contract unless supplemental funds are provided.

We request that supplementary funds of $94,000 be allocated to this Laboratory for its role in the collaborative studies with Microbiological Associates. A summary of responsibilities and costs and an initial protocol for the dosimetry experiment are appended.

M. R. Guerin

M. R. Guerin
Director, Tobacco Smoke Research Program
Analytical Chemistry Division

J. R. Stokely

J. R. Stokely
CTR-USA/ORNL Contract
Principal Investigator

MRG:JRS:scr

Attachment

cc: J. C. White
W. D. Shults
J. E. Carr
J. E. Caton
H. R. Beatty

1003536289

Responsibilities and Cost

An outline of variables to be tested and of the experimental protocol developed by Dr.'s Stokely (ORNL) and Whitmire (Microbiological Assoc.) is appended. For each set of variables tested, conditioned animals are exposed to smoke from a tracer-containing cigarette, the animals are immediately sacrificed and the target organs/tissues are analyzed for tracer content. The quantity of tracer found is converted to quantity of smoke particulates using the pre-determined specific activity of the particulates.

The responsibilities of this Laboratory are:

(1) Preparation of Radiolabelled Cigarettes
- (a) Weight and Pressure Drop Selection
- (b) Spiking with Tracer
- (c) Quality Control - specific activity of TPM, determination of TPM, water, nicotine, tar

(2) Analysis of Resulting Samples
- (a) Tissues, Organs, etc. from Exposed Animals - Carbon 14
- (b) Input filters taken prior to exposure--carbon 14, nicotine, water, tar
- (c) Chamber sample taken during exposure--nicotine, carbon 14
- (d) Input and/or Chamber Samples taken to monitor machine--nicotine, water, tar

(3) Information, Hardware, and Technology Transfer
- (a) Construction and installation of synchronized chamber sampler
- (b) Computerized handling of dosimetry data--processing, statistical analysis, study, reporting
- (c) Transfer of radiolabelled cigarettes, radioactive samples
- (d) On-site instruction and set up--input and chamber sampling, TPM determination, sample preparation, etc.

Using the projected (see appended protocol) numbers of samples as a guide, allowing for an additional 15% in the number of samples for experiments which must be repeated and an additional 15% for experiments using a continuous smoke generator, personnel/budget requirements are as follows.

1003536290

1. Personnel (85,000)

(a) Technician (1.5 manyears). Analysis of tissues and filters for carbon-14, tracer, cigarette selection

(b) Chemist, B.S. level (0.75 manyear). Analysis of filter samples for nicotine and water, quality control analyses, radiolabelled cigarette preparation, coordinate technicians, data handling.

(c) Chemist, Ph.D. level (0.25 manyear). Initial set-up, liaison with collaborators, data study, person-in-charge.

2. Special Supplies (3,900)

(a) Carbon-14 dotriacontane tracer (for 600 cigarettes) 900

(b) Analytical supplies (liquid scintillation supplies, solvents, standards, chromatographic supplies) 3,000

3. Miscellaneous (5,100)

(a) Travel (4 visits to Microbiological Associates by 2 scientists; 8 man-trips) 2,000

(b) Synchronized Chamber Sampler 1,100

(c) Shipping Radioactive Samples 2,000

TOTAL (94,000)

1003536291

PROTOCOL FOR MOUSE DOSIMETRY EXPERIMENTS ON WALTON HORIZONTAL SMOKING MACHINE

Prepared by

C. E. Whitmire
Microbiological Associates

and

J. R. Stokely
Oak Ridge National Laboratory

Date: September 16, 1974

This protocol outlines proposed collaborative studies by Microbiological Associates and Oak Ridge National Laboratory to investigate mouse dosimetry on the Walton Horizontal Smoking Machine. The objectives of these studies are:

(1) To define the dose of tobacco smoke particulates received by mice under selected exposure conditions (exposure time and smoke concentration),

(2) To ascertain possible effects of sex and strain on dosimetry so that a rational selection can be made for future studies of biological impact,

(3) To determine retention and clearance rate of smoke particulates after exposure, and

(4) To determine cumulative dose and long-term retention of smoke particulates under typical exposure regimes.

Responsibilities of Oak Ridge National Laboratory are the following:

(1) Weight and RTD selection of cigarettes.
(2) Labeling of cigarettes with ^{14}C tracers.
(3) Quality control of cigarettes (determination of nicotine, TPM, tar, and ^{14}C delivery of selected radiolabeled cigarettes).
(4) Shipping of radiolabeled cigarettes to Microbiological Associates.
(5) Construction and testing of apparatus for sampling exposure chamber.
(6) Instruction and assistance on operation of smoking machines, sampling apparatus, and cigarette conditioning and handling.
(7) Analysis of animal tissues for ^{14}C tracers.
(8) Analysis of input Cambridge filter pads for nicotine and ^{14}C tracer and chamber samples for ^{14}C tracer.
(9) Calculations to obtain the following results:
    (a) absolute activity (dpm) of tracer in each tissue specimen.

1003536292

Responsibilities of ORNL (continued)

(9) (b) absolute tar (mg) deposited in each tissue specimen based on tracer deposition.
- (c) percent distribution of activity among various tissue specimens for each animal.
- (d) dose expressed as percentage of smoke input to exposure chamber.

Responsibilities of Microbiological Associates are the following:

(1) Calibration of smoking machines (puff volume, puff time, exposure time, purge time).
(2) Conditioning and weighing of labeled cigarettes. After receipt from Oak Ridge National Laboratory.
(3) Collection of samples of smoke input to smoking machines and chamber samples obtained during animal exposures.
(4) Animal conditioning.
(5) Animal exposure.
(6) Animal sacrifice and dissection.
(7) Shipping of tissue specimens, input filter pads, and chamber samples to Oak Ridge National Laboratory.

Experiment I. Effect of Exposure Time

One mouse strain: C3H/fMai

One sex: female

Four exposure time intervals: 10, 20, 30, 40 seconds

One exposure concentration: 10%

Four tissue samples: skinned head, upper trachea and larynx, lungs and lower half of trachea, stomach and esophagus

Number of mice per exposure: 10 (plus 10 scrubs)

Number of cigarettes per exposure: 3 (2 for chamber input, 1 for exposure)

Chamber samples per exposure: 1

Cigarette type: Kentucky Reference 1A1 loaded with 5×10^6 dpm ^{14}C-dotriacontane--weight and RTD tested ($\pm$ 20 mg, $\pm$ 5 mm H_2O)

Number of repetitive exposures: 4 (40 mice)

Instant sacrifice

Tissue specimens: 640

Total Cigarettes: 60 (assume 25% loss).

Experiment II. Effect of Smoke Concentration

One exposure time interval: based on experiment I

Five exposure concentrations: 20% (2 cigarettes--384 ml chamber),
30% (3 cigarettes--384 ml chamber),
5% (1 cigarette--768 ml chamber),
10% (2 cigarettes--768 ml chamber),
15% (3 cigarettes--768 ml chamber)

Number of cigarettes per exposure: 6 (20%), 9 (30%), 3 (5%), 6 (10%), 9 (15%)

Other conditions: same as experiment I

Total tissue specimens: 800

Total cigarettes: 165 (assume 25% loss).

Experiment III. Effect of Sex

Two sex: male and female

One exposure time interval: based on experiment I

One exposure concentration: based on experiment II

Number of mice per exposure: 20 (10 male, 10 female)

Other conditions: same as experiment I

Tissue specimens: 300

Total cigarettes: 15 (assume 25% loss).

Experiment IV. Strain Differences

Four mouse strains: C3H/f, C57BL/6, DBA/2, BC3F1

Sex: based on experiment III (male, female, or both)

One exposure time: based on experiment I

One exposure concentration: based on experiment II

Four tissue samples: experiment I

Number of mice per exposure: 20 (10 each of 2 strains or sexes)

Other conditions: same as experiment I

Tissue specimens: 640 (1 sex)
1280 (2 sexes)

Number of cigarettes: 30 (1 sex)
60 (2 sexes) (assume 25% loss).

Experiment V. Retention Period of ^{14}C-Dotriacontane

One mouse strain: C3H/f

One sex: based on experiments III and IV

One exposure time: based on experiment I

One exposure concentration: based on experiment II

Five tissue samples per animal: same as experiment I plus composite sample of all other animal tissues--animal skinned--skin not included in composite.

Experiment V (continued)

Number of mice per exposure: 20

Five sacrifice times after smoking: 0.25, 1, 4, 16, 24 hours. Four animals sacrificed at each time.

Number of cigarettes per exposure: 3

Cigarette type: 1A1 loaded with maximum amount (up to 1×10^8 dpm) ^{14}C-dotriacontane.

Number of repetitive exposures: 4

Tissue specimens: 400

Number of cigarettes: 15 (assume 25% loss).

Experiment VI. Retention Period of ^{14}C-Benz(a)pyrene

Cigarette type: 1A1 loaded with maximum amount (up to 1×10^8 dpm) ^{14}C-benz(a)pyrene

Other conditions: same as experiment V

Tissue specimens: 400

Number of cigarettes: 15 (assume 25% loss).

Experiment VII. Retention Period of ^{14}C-Nicotine

Five sacrifice times after smoking: immediately, 0.25, 0.5, 1, 4 hours after smoking. Four animals sacrificed at each time.

Cigarette type: 1A1 loaded with maximum amount (up to 1×10^8) ^{14}C-nicotine

Other conditions: same as experiment V.

Tissue specimens: 400

Number of cigarettes: 15 (assume 25% loss).

Experiment VIII. Comparative Retention Periods for Two Mouse Strains

Two strains: based on experiment IV

One sex: based on experiments III and IV

Experiment VIII (continued)

One exposure time: based on experiment I

One exposure concentration: based on experiment II

Two retention periods: based on experiments V, VI, and VII

Five tissue specimens per animal: experiment V

Twenty mice per exposure: ten mice sacrificed at each retention period

Number of repetitive exposures: 2

Three type cigarettes: Kentucky reference 1A1 loaded with ^{14}C-nicotine, ^{14}C-benz(a)pyrene, or ^{14}C-dotriacontane (maximum load)

Tissue specimens: 1200

Number of cigarettes: 15 (assume 25% loss).

Experiment IX. Cumulative Dose and Long Term Retention

Number of radiolabeled cigarettes given per 8 hour day: 2, 5, 10, 20

One strain - based on experiment VIII

Four animals sacrificed at each of the following times after exposure: immediately, 4, 24, 48, 120 hours

Three type cigarettes: same as experiment VIII

Number of repetitive tests: 1

Other conditions: same as experiment VIII

Tissue specimens: 1200

Number of cigarettes 169 (assume 25% loss).

1003536297

Summary of Tissue Specimens and Number of Cigarettes

Experiment	Tissue Specimens[1]	Number of Cigarettes[2]
I	640	60
II	800	165
III	320	15
IV	1280	60
V	400	15
VI	400	15
VII	400	15
VIII	1200	15
IX	1200	169
	6640	529

[1] Maximum

[2] Assume 25% loss

1003536298

ANIMAL CARCINOGENESIS MODEL

1003536299

WHITMIRE - M.A.

1003536300

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

September 17, 1974

To: The Scientific Advisory Board

Subject: Renewal Request: Mouse Model System for In Vivo Lung Carcinogenesis
CTR Contract #2 (MA #2220)

This pilot project is designed to further define a mouse carcinogenic model system which can be used for further inhalation studies. Some promising leads have been confirmed, i.e., that squamous cell carcinoma can be induced in an AHH inducible mouse, following intratracheal injection of M.C.A.

The experiment #5 designed to elucidate the relationship between sensitivity to intratracheally instilled M.C.A. induced squamous cell carcinomas and inducibility of AHH is currently being repeated (CTR #39) using the C_3H and DBA lines and appropriate crosses. This cross mimics the autosomal codominant relationship reported by Shaw in humans. The original study (CTR #5) using the $C_{57}B16$ X DBA_2 cross was not effective in inducing significant numbers of squamous cell carcinomas, possibly due to small particle size.

Particle size, or chemical residence time in the lung, may also explain why an M.C.A. dose in a gelatin vehicle is much more lethal than M.C.A. in a treoctanoine carrier (CTR 3A, 3B) and more carcinogenic (CTR 3A, 3B, 3C) in AHH inducible mice.

An additional study to be initiated within the next few weeks is a study of vitamin A in carcinogenesis, with squamouse cell carcinoma of the lung as an end point.

The studies proposed using dioxime (TCDD) as an inducer of all types of AHH enzymes (constituitive and induced), and the studies of AHH competitive inhibitors and their effects on M.C.A. induced lung tumors are directed to answering questions concerning chronicity of exposure to AHH inducers (such as is the case of a heavy smoker) and potentiation of lung tumorigenesis. Preliminary evidence of optimal tumor production conditions points to a necessity for concurrent exposure to AHH inducer and carcinogen. This must be repeated and documented. Inhibition studies at various steps in the metabolic breakdown of a polycyclic aromatic hydrocarbon should help to further elucidate the mechanism of carcinogenesis by PAH chemical carcinogens.

Smoke condensate dissolved in beeswax pellets and implanted S.C. resulted in insignificant tumors. However, tumors were readily

1003536301

produced when 10 mg M.C.A. (normally below the tumor breakthrough level) plus smoke condensate fractions were mixed in a beeswax pellet. Further work including subfractionations will be required to show the mode of action of specific fractions, whether as enzyme inducers, DNA repair inhibitors or other.

An experiment has been added to the list after the August 19 deadline. This experiment is a criticallt important dosimetry study to show the lowest levels of M.C.A. and BP which, if instilled, can produce tumors. This must precede inhalation studies with smoke as cocarcinogen.

J.H.K.

JHK:wg

1003536302

DEVELOPMENT OF A MOUSE MODEL SYSTEM FOR
IN VIVO LUNG CARCINOGENESIS

CTR Contract # 2
MA Contract 2220

PROGRESS REPORT
AND
CONTRACT RENEWAL PROPOSAL
FOR THE PERIOD
JAN.1,1975 - DEC.31,1975

1003536303

August 27,1974

Vincent L. Ruwet

Vincent L. Ruwet
Vice-President, Contracts and
Administration

TO: Council for Tobacco Research
110 East 59th Street
New York, N.Y. 10022

FROM: Microbiological Associates, A Division
of Dynasciences Corporation
4733 Bethesda Avenue
Bethesda, Maryland 20014

DATE: August 27, 1974

1003536304

DEVELOPMENT OF A MOUSE MODEL SYSTEM FOR
IN VIVO LUNG CARCINOGENESIS

There is a real need for the determination of those factors which alter or influence the biological effects of cigarette smoking. A model system analogous to the human situation must be devised. We feel that the best model system presently available involves the use of inbred strains of mice, for the genetics, biochemistry, and types of tumor responses of this model system closely approximate the condition in humans. This system is also economically feasible. To this end we now present a series of experiments which should crystallize our concepts of this model system and thus lay the groundwork for a large scale inhalation program designed to finally establish many of the risks involved in cigarette smoking.

Prepared by

Carrie E. Whitmire, Ph.D.
Co-Project Director

Richard E. Kouri, Ph.D.
Co-Project Director

Charles F. Demoise, Ph.D.
Associate Project Director

Bernard Sass, D.V.M.
Pathologist

TABLE OF CONTENTS

1003536306

1003536307

1003536308

Summary Report

I. SUMMARY PROGRESS REPORT

The experiments reported on under this contract are those described in the proposal for the contract year. The volume of work however does not reflect only those expenditures under this contract but rather also includes effort and expenditures under the larger contract running from July 1-June 30 each year. Of necessity we have attempted to have an integrated CTR program thus the work is treated as one large scientific effort although it is funded under several contracts.

The CTR-MA contracts have two basic functions:
First, to define a mouse carcinogenic model system which can be used for future inhalation studies.
Second, to undertake screening of known carcinogens, cigarette smoke condensates and condensate fractions by subcutaneous and pulmonary inoculations of mice.

A. Model System

1. Chemical Carcinogensis

a. MCA - SC Route

In order to define the mouse model systems, we have undertaken subcutaneous carcinogenesis studies with MCA to determine the relative sensitivity of several mouse strains as quickly as possible, and to compare subcutaneous and intratracheal susceptibility. This MCA-SC study (CTR-4) has been completed. Based on the MCA dose required to produce fifty percent tumors (TD_{50}) in eight months, the most susceptible strain was the C3H/f, followed by the B6C3F1 hybrid using the C3H/fMai and the C57BL/6 Cum mice. This hybrid is not available commercially but should be considered as a possible susceptible strain after it is characterized for other carcinogens. The time required for tumor development more closely paralleled that of the C3H parent. All the strains were AHH inducible. The C57BL and C57BL/6 mice were virtually negative for type C RNA gs antigen, while nearly all the C3H/f mice were gs +. The hybrid mice appear to take on the same gs antigen expression as the C3H/f parent.

b. MCA-IT Route

These same stains (C57BL, C57BL/6, C3H/f and BC3F1) have been given intratracheal inoculation of MCA (CTR-3). These mice have been on test for approximately 72 weeks. Although we lost a large number of mice during the inoculation period, we have seen very few lung tumors in mice sacrificed at varying time intervals. Within the past week we have seen several large lung tumors, however, we do not have histopathology at this time. Additional mice have been inoculated (CTR-3B, C, D) and will be observed

for tumor induction. Recent reports of the histopathology indicate that better tumor induction can be accomplished with MCA which is not sonicated so extensively, therefore, larger particles are instilled. It appears that lower doses and inoculation at two week intervals may improve survival of the mice with less pneumonia.

To study the effects of AHH inducibility on the induction of lung tumors by MCA intratracehal instillation, C57BL/6, DBA/2 and the hybrid mice have been inoculated (CTR-5). These mice have been on test up to 17 months and few tumors have been found in the few which have been sacrificed. The same problems exist with this experiment as our original CTR-3 in that the mice received MCA of too fine particle size and at too high a dose which produced early deaths. This will be repeated as CTR-39.

c. Chemical Carcinogens (Nitrosamines) (SC&IT Routes)

In defining the model system it is essential to determine the susceptibility of our selected mouse strains to nitrosamine carcinogenesis, since they are present in tobacco smoke. Three routes of administration have been studied. We inoculated neonate (less than 5 days of age) C57BL/6Cum mice intraperitoneally in an effort to induce lung and bladder tumors. After eight months, some mice treated with DMN have developed liver and lung tumors and the tumors have been submitted for histopathology. Additional mice are on test with DMN, DEN, DBN, PIP and PYR (CTR-2, 2A). Other studies have been undertaken to determine if nitrosamine can produce lung tumors when given intratracheally (CTR-18). To demonstrate whether there is any difference in susceptibility of AHH inducible mice and the non-inducible mice several strains have been injected into the lung with wax pellets containing DMN.

d. AHH Inducers

Studies were undertaken using a chemical (TCDD) which induces AHH regardless of genotype (CTR-15, 16, 17). Considerable toxicity was encountered. Both C57BL/6 and DBA/2 mice were tested to determine the effects of TCDD on subcutaneous tumor induction. The results with the TCDD are compatible with the idea that AHH induction (via TCDD) simultaneous with MCA treatment yields more tumors than MCA alone, but the results with dioxane are difficult to assess. Why a 48 hr. pretreatment with 0.010 ml dioxane should enhance MCA-induced tumorigenesis cannot be explained at this time. The fact that both the low and high TCDD levels, when given 48 hrs. before MCA, had no effect, yet dioxane was also in these treatments, indicates that whatever the effect of dioxane, it is cancelled, if TCDD is present.

We feel that a repeat of this experiment must be done. The protocol for this repeat is CTR-40.

2. Screening of Carcinogens

a. Subcutaneous Route

Earlier studies with MCA, DMBA and BP have shown the C3H/fMai strain to be the most susceptible to subcutaneous carcinogenesis. Based on these studies we undertook the screening of 14 fractions of 1R1 and 1A1 reference cigarette smoke condensates to determine their ability to induce tumors alone or with MCA as a co-carcinogen. In order to give as high doses of these fractions as possible, they were implanted as pellets using 1:1 beeswax:trioctanoin as a vehicle. This vehicle has advantages as well as disadvantages. Although it allows larger doses to be given without toxicity, the latency period for tumor development is extended. MCA (150μg) in trioctanoin induced 100% tumors in 20 weeks; while 150μg in beeswax:trioctanoin has produced only 43% tumors in 80 weeks. When 10μg MCA was given in trioctanoin, 38% tumors developed in 65 weeks; however, no tumors developed in the same period when 10μg MCA was given in beeswax:trioctanoin. When 10μg MCA in trioctanoin was injected directly into the fresh beeswax:trioctanoin pellet, as was done with the CSC fraction, 13% tumors have developed in 65 weeks. Further studies to define the relationship between vehicle, carcinogen dose and tumor latency are planned (CTR 19) using known carcinogens.

The 1R1 fractions have been on test for 82 weeks. Several tumors have developed with the fraction alone, however, with MCA (10μg) as a co-carcinogen as high as 75% tumors have been induced with the N_{CH} fraction of 1R1. The 1A1 fractions have also been tested in a similar manner. No tumors have developed with the CSC fraction alone however with 10μg MCA we have obtained up to 50% tumors. These studies with 1A1 have been on over 66 weeks. At this time, they appear to be less co-carcinogenic than the 1R1 fractions.

Fifteen whole cigarette smoke condensates were obtained through Dr. Gori at NCI and have been on test for 14 months (CTR-1B). No tumors have occurred in mice receiving only the condensates. As co-carcinogens with 10μg MCA, tumor incidences range from 0 - 67%, while 13% tumors have occurred with the 10μg MCA control.

1003536311

1003536312

Progress Repor

August 19, 1974

CTR-1 Carcinogenic and Co-Carcinogenic Subcutaneous Studies with 1R1 Cigarette Smoke Condensate (CSC) Fractions in C3H/fMai Mice.

Objective:

To determine the complete and co-carcinogenic potential of 14 fractions of 1R1 cigarette smoke condensate by our most rapid subcutaneous mouse tumor assay system.

Procedure:

Various fractions of 1R1-CSC were diluted (w/v) in a 1:1 mixture of trioctanoin:beeswax and inoculated subcutaneously in C3H/fMai mice. These fractions were tested in the presence of, and without, 10 µg MCA delivered at the same site of inoculation.

Progress:

1. Mice that received subcutaneous wax-implants containing either 1R1 fractions of CSC or 10 µg of 3-methylcholanthrene (MCA) or both (co-carcinogenic) have been on test from 73 to 82 weeks. Tumor incidence and latency periods for various fractions tested are presented in Table 1. In all but two cases, tumors occurred only in mice that received both MCA and CSC fractions (co-carcinogenic). The highest tumor incidences were in fractions N_CH (75%), N_{MeOH} (53%), WA_I (50%), B_E (50%), St.Mat. (50%), and B_Ia (47%). The tumor latency periods ranged from 27.8 to 49.9 weeks for those mice that received both MCA and CSC fractions. Since only a few mice remained on test the experiment has been terminated. Histopathological studies on induced tumors are in progress.

1003536313

CTR-1 Table 1: Tumor incidence and latency period for 1R1 fractions

Fraction tested[a]	Dose (mg)	% of CSC	w or w/o 10 µg MCA[b]	wks. on test	Tumor Incidence[c]		Avg. Latency Period (wks)[d]
St. Mat.	10.0	100.00	-	82	0/20	0	-
			+		8/16	50%	38.7
Rec. Mat.	10.0	97.16	-	82	0/20	0	-
			+		10/20	50%	28.8
B_Ib	5.0	0.39	-	82	1/7	14%	82.0
			+		Not done	-	-
B_Ia	5.0	0.99	-	82	1/8	5%	65.3
			+		9/19	47%	27.8
B_E	0.5	8.10	-	73	0/20	0	-
			+		10/20	50%	29.2
B_W	2.5	2.79	-	73	0/20	0	-
			+		5/14	36%	49.9
WA_I	10.0	6.52	-	82	1/20	6%	51.1
			+		6/12	50%	43.0
WA_E	10.0	7.50	-	82	0/20	0	-
			+		1/19	5%	34.8
SA_I	5.0	1.77	-	82	0/20	0	-
			+		8/20	40%	31.0
SA_E	10.0	3.30	-	82	0/20	0	-
			+		8/20	40%	28.6
SA_W	10.0	40.50	-	80	0/20	0	-
			+		6/19	32%	32.3
N_{MeOH}	10.0	4.50	-	82	0/20	0	-
			+		10/19	53%	31.8
N_{CH}	10.0	18.10	-	82	0/20	0	-
			+		12/16	75%	31.6
N_{NM}	10.0	2.70	-	82	0/20	0	-
			+		7/17	41%	36.3
150 µg MCA/BW:T	-		+	80	8/19	43%	27.4
150 µg MCA/Trioc	-		+	20	18/18	100%	10.2
10 µg MCA/BW:T	-		+	64	0/46	0%	-
10 µg MCA/Trioc	-		+	65	19/50	38%	29.0
10 µg MCA injected into BW pellet	-		+	65	6/48	13%	46.0

[a] One part of cigarette smoke fraction or chemical carcinogen was combined with a warm (78°C) mixture of Beeswax-trioctanoin (1:1) at the dilution indicated for subcutaneous inoculation.

[b] 10 µg of MCA dissolved in trioctanoin delivered at the same site that cigarette fraction was administered.

[c] Tumor incidence is the current number of tumors divided by the number of mice on test when the first tumor occurred.

[d] Latency period is the total number of weeks prior to the appearance of a tumor divided by the total number of tumors in a particular test group.

1003536314

August 19, 1974

CTR-1A: Carcinogenic and Co-Carcinogenic Subcutaneous Studies with 14 Fractions of 1A1 Tobacco Smoke Condensate in C3H/fMai Mice.

Objective:

To determine the complete and co-carcinogenic potential of 14 fractions of 1A1 cigarette smoke condensate by our most rapid subcutaneous tumor assay system.

Procedure:

Various fractions of 1A1-CSC were diluted (w/v) in a 1:1 mixture of trioctanoin;beeswax and inoculated subcutaneously in C3H/fMai mice. These condensates were tested in the presence of, and without, 10 µg MCA delivered at the same site of inoculation.

Progress:

1. Mice have been on test approximately 66 weeks and tumors have occurred only in mice that received both MCA and 1A1 fractions (co-carcinogenic). Tumor incidence ranges from 5 to 50%, with fractions BI^b (50%) and N_{MeOH} (40%) being the highest. Latency periods ranged from 22 to 41 weeks. This experiment will be terminated at 82 weeks.

Conclusion:

The fractions of 1R1 appears to give more tumors than 1A1 when administered subcutaneously as a cocarcinogen with 10µg MCA (See Table 2).

1003536315

August 12, 1974

CTR-1A Table 1: Tumor incidence and latency period for 1A1 fractions

Fraction tested[a]	Dose (mg)	% of CSC	w or w/o 10 μg MCA[b]	Wks. on test	Tumor Incidence[c]		Latency Period (wks)[d]
St. Mat.	10.0	100.00	-	67	0/18	0	
			+		3/20	15%	41
Rec. Mat.	10.0	97.16	-	66	0/16	0	
			+		4/20	20%	38
B_Ib	5.0	.39	-	66	0/14	0	
			+		7/14	50%	23.4
B_Ia	5.0	.99	-	66	0/17	0	
			+		5/20	25%	32.3
B_E	1.0	8.10	-	65	0/13	0	
			+		3/20	15%	35
B_W	2.5	2.79	-	67	0/15	0	
			+		2/20	10%	34
WA_I	10.0	6.52	-	67	0/18	0	
			+		5/20	20%	30
WA_E	10.0	7.50	-	67	0/21	0	
			+		4/20	20%	22.1
SA_I	5.0	1.77	-	66	0/18	0	
			+		4/20	20%	41
SA_E	10.0	3.30	-	66	0/15	0	
			+		3/20	15%	39.3
SA_W	10.0	40.50	-	68	0/12	0	
			+		1/21	5%	31
N_{MeOH}	10.0	4.50	-	66	0/20	0	
			+		8/20	40%	39
N_{CH}	10.0	18.10	-	66	0/20	0	
			+		2/20	15%	36.4
N_{NM}	10.0	2.70	-	66	0/18	0	
			+		1/20	5%	30.4
10 μg MCA/.05 ml trioc			+	65	19/50	38%	29
10 μg MCA/.05 ml trioc + BW:T			+	65	6/48	13%	46
10 μg MCA/.05 ml BW:T			+	64	0/46	0	
150 μg MCA/.05 ml trioc			+	20	13/13	100%	14
150 μg MCA/.05 ml BW:T			+	64	12/20	60%	24

[a,b,c,d] See footnotes for CTR-1, Table 1.

1003536316

CTR-1, 1A: — August 19, 1974

Table 2. Co-Carcinogenic Subcutaneous Pellet Studies with 1R1 and 1A1 Smoke Condensate Fractions* in C3H/fMai Mice.

CSC Stedman No. (Fraction)	CSC %	Dose** (mg)	% Tumor Incidence (65 Weeks) 1R1 0µg MCA	1R1 10µg MCA	1A1 0µg MCA	1A1 10µg MCA	Carcinogenic Ratio*** 1R1	Carcinogenic Ratio*** 1A1
1 (St.Mat.)	100.00	10.0	0	50	0	15	4.0	1.2
2 (Rec.Mat.)	97.16	10.0	0	50	0	20	4.0	1.6
3 (B_Ia)	.99	5.0	0	47	0	25	2.7	2.0
4 (B_Ib)	.39	5.0	0	N.D.	0	50	N.D.	4.0
5 (B_E)	8.10	0.5	0	50	0	15	4.0	1.2
6 (B_W)	2.79	2.5	0	36	0	10	2.9	0.8
7 (WA_I)	6.52	10.0	0	50	0	25	4.0	2.0
8 (WA_E)	7.50	10.0	0	5	0	20	0.4	1.6
9 (SA_I)	1.77	5.0	0	30	0	20	2.4	1.6
10 (SA_E)	3.30	10.0	0	40	0	15	3.2	1.2
11 (SA_W)	40.50	10.0	0	32	0	10	2.6	0.8
12 (N_{MeOH})	4.50	10.0	0	63	0	40	5.0	3.2
13 (N_{CH})	18.10	10.0	0	75	0	15	6.0	1.2
14 (N_{MN})	2.70	10.0	0	41	0	5	3.3	0.4

Controls:

trioctanoin:beeswax 0/46 0% tumor incidence at 65 wks.
10µg MCA in trioc:BW 0/46 0% tumor incidence at 64 wks.
10µg MCA/trioctanoin 19/50 38% tumor incidence at 65 wks.
10µg MCA into trioctanoin:beeswax 6/48 13% tumor incidence at 65 wks.
150µg MCA/trioctanoin 13/13 100% tumor incidence at 20 wks.

*Smoke Condensate Fractions Prepared by Dr. A.R. Patel, Meloy Labs, Inc.

**Dose was dependent on toxicity of material given subcutaneously

***Carcinogenic Ratio = $\frac{\text{\% Tumors Induced with Fraction + 10µg MCA}}{\text{\% Tumors Induced with 10µg MCA}}$

1003536317

August 19, 1974

CTR-1B: Carcinogenic and Co-Carcinogenic Studies with Cigarette Smoke Condensate (CSC) Inoculated Subcutaneously in C3H/fMai Mice.

Objective:

To determine the carcinogenic and co-carcinogenic properties of various cigarette smoke condensates (CSC) in C3H/fMai mice when administered subcutaneously.

Procedure:

Various samples of CSC from Meloy Labs were diluted 1:5 (w/v) in a 1:1 mixture of trioctanoin:beeswax and inoculated subcutaneously in C3H/fMai mice. These condensates were tested with and without 10 µg MCA delivered at the same site.

Progress:

1. Wax pellets of cigarette smoke condensates were given subcutaneously to mice with and without 3-methylcholanthrene. These mice have currently been on test about 58 weeks (see attached Table 1.).

2. No tumors have been observed among mice that received only cigarette condensates. However, condensates plus MCA (co-carcinogenic) gave tumor incidences that ranged from 0% (condensate #57) to 67% (condensate #60). The mean latency period for tumors in cocarcinogenic mice ranged from 17 weeks (condensate #61) to 48 weeks (condensate #55).

1003536318

CTR-1B: Carcinogenic and Co-Carcinogenic Studies with Cigarette Smoke Condensates Inoculated Subcutaneously in C3H/fMai Mice

Condensate Tested[a]	Dose (mg)	± MCA[b] 10 µg	Wks. on Test	Tumor Incidence[c]		Latency Period[d]
40	10	-	59	0/7	0	-
		+	59	2/14	14	29.5
41	10	-	59	0/18	0	-
		+	59	6/14	42	32.8
42	10	-	59	0/11	0	-
		+	59	7/13	54	32.0
51	10	-	59	0/8	0	-
		+	59	1/6	16	48
52	10	-	59	0/13	0	-
		+	59	3/19	16	36.3
53	10	-	59	0/15	0	-
		+	59	5/17	29	31.3
54	10	-	59	0/13	0	-
		+	59	6/20	30	32.3
55	10	-	58	0/19	0	-
		+	58	2/19	11	19
56	10	-	58	0/20	0	-
		+	58	7/19	37	38.6
57	10	-	58	0/11	0	-
		+	58	0/8	0	-
58	10	-	58	0/17	0	-
		+	58	6/18	33	34.2
59	10	-	58	0/16	0	-
		+	58	5/19	26	36.4
60	10	-	58	0/8	0	-
		+	58	6/9	67	40.0
61	10	-	58	0/12	0	-
		+	58	1/8	13	17
62	10	-	58	0/15	0	-
		+	58	6/17	35	39.3
57	5	-	57	0/5	0	-
		+	57	3/10	30	31.3
60	5	-	57	0/9	0	-
		+	57	2/6	33	33.5
61	5	-	57	0/6	0	-
		+	57	2/10	20	33.5
10µg MCA/.05 ml trioc		+	65	19/50	38	39 wks.
10µg MCA/.05 ml trioc into BW:T pellet		+	65	6/48	13	46 wks.
10µg MCA/.05 ml BW:T		+	64	0/46	0	-
150µg MCA/.05 ml trioc		+	20	13/13	100	14 wks.
150µg MCA/.05 ml BW:T		+	64	12/20	60	24 wks.

a,b,c,d See footnotes for CTR-1, Table 1.

August 19, 1974

1003536319

August 19, 1974

CTR-2: Potential Carcinogenic Effect of Nitrosamines in C57BL/6Cum mice.

Objective:

Preliminary studies were carried out to determine the toxicity of various nitrosamines and their carcinogenicity in C57BL/6Cum mice.

Procedure:

Intraperitoneal (IP) inoculation of newborn C57BL/6Cum mice for acute toxicity and carcinogenicity.

Progress:

1. This experiment has been a pilot study to determine the toxicity and potential carcinogenicity of various levels of nitrosamines in newborn C57BL/6Cum mice. This experiment has been terminated after 12 months testing and the available results are presented in the following tables.

Table 1. Effects of nitrosamines on lung histology.
Table 2. Effects of nitrosamines on liver.
Table 3. Liver histology at 12 months after nitrosamine exposure.

Toxicity effects of various doses of nitrosamines were reported in the early June update summary. Few mice survived for necropsy, but those that did have been evaluated and the results are shown in the accompanying tables.

2. This experiment (CTR-2) has been repeated as CTR-2A which has now been on test about 39 to 49 weeks. Gross and histopathological observations will be performed at 12 or 15 months on these animals.

August 8, 1974

CTR-2 Table 1: Effects of Nitrosamines on Lung Histology

Material[a] Tested	Diagnosis	Months after Exposure 6	10-11	12
dimethyl-nitrosamine .05 mg	BA lesion	3/23(13%)[b]	0/13(0%)	2/25(8%)
	adenomata	1/23(4%)	4/13(31%)	5/25(20%)
	hyperplasia	0/23(0%)	0/13(0%)	n.a.
	inflamation	0/23(0%)	0/13(0%)	2/25(8%)
	pneumonia	1/23(4%)	1/13(8%)	2/25(8%)
	lymphocyte accum.	n.a.	n.a.	
diethyl-nitrosamine .01 mg	BA lesion	1/36(3%)	0/14(0%)	1/24(4%)
	adenomata	0/36(0%)	4/14(29%)	8/24(33%)
	hyperplasia	1/36(3%)	0/14(0%)	n.a.
	inflamation	0/36(0%)	0/14(0%)	0/24(0%)
	pneumonia	0/36(0%)	2/14(14%)	0/24(0%)
	lymphocyte accum.	n.a.	n.a.	3/24(12%)
dibutyl-nitrosamine 40 mg	BA lesion	10/26(39%)		2/53(4%)
	adenomata	1/26(4%)		13/53(25%)
	hyperplasia	0/26(0%)		n.a.
	inflamation	0/26(0%)		2/53(4%)
	pneumonia	4/26(15%)		8/53(15%)
	lymphocyte accum.	n.a.		5/53(9%)
N-nitroso-piperidine .05 mg	BA lesion	1/16(6%)	3/13(23%)	0/33(0%)
	adenomata	0/16(0%)	1/13(8%)	6/33(18%)
	hyperplasia	2/16(13%)	0/13(0%)	n.a.
	inflamation	0/16(0%)	0/13(0%)	0/33(0%)
	pneumonia	2/16(13%)	0/13(0%)	1/33(3%)
	lymphocyte accum.	n.a.	n.a.	2/33(6%)
N-nitroso-pyrrolidine .13 mg	BA lesion	2/26(8%)	2/13(15%)	1/22(5%)
	adenomata	0/26(0%)	0/13(0%)	2/22(9%)
	hyperplasia	2/26(8%)	1/13(8%)	n.a.
	inflamation	0/26(0%)	2/13(16%)	0/22(0%)
	pneumonia	3/26(12%)	0/13(0%)	0/22(0%)
	lymphocyte accum.	n.a.	n.a.	0/22(0%)
Trioctanoin .05 ml	BA lesion		1/4(25%)	1/12(8%)
	adenomata		0/4(0%)	0/12(0%)
	hyperplasia		0/4(0%)	0/12(0%)
	inflamation		0/4(0%)	0/12(0%)
	pneumonia		0/4(0%)	0/12(0%)
	lymphocyte accum.		n.a.	n.a.

1003536321

[a] Various nitrosamines dissolved in trioctanoin and given by intra-peritoneal injection to newborn C57BL/6Cum mice.

[b] Number of mice positive divided by number examined.

CTR-2 Table 2: Effects of Nitrosamines on Liver

Material Tested[a]	Liver Status	6		10		11		12	
		Months After Exposure							
dimethylnitrosamine .05 mg	abnormal[b]	8/12	(67%)			11/12	(85%)	9/12	(75%)
	tumored[c]	5/12	(42%)			4/13	(31%)	8/12	(67%)
diethylnitrosamine .01 mg	abnormal	2/24	(8%)	2/15	(13%)			6/24	(25%)
	tumored	2/24	(8%)	0/15	(0%)			3/24	(13%)
dibutylnitrosamine .40 mg	abnormal	2/26	(8%)					22/53	(42%)
	tumored	0/26	(0%)					14/53	(26%)
N-nitrosopiperidine .05 mg	abnormal	1/6	(17%)	2/13	(15%)			3/33	(9%)
	tumored	0/6	(0%)	0/13	(0%)			0/33	(0%)
N-nitrosopyrrolidine .13 mg	abnormal	1/14	(7%)	0/13	(0%)			1/22	(5%)
	tumored	0/14	(0%)	0/13	(0%)			0/22	(0%)
Trioctanoin .05 ml	abnormal					0/4	(0%)	1/8	(13%)
	tumored					0/4	(0%)	0/8	(0%)

[a] Various nitorsamines given by intraperitoneal injection to newborn C57BL/6Cum mice.

[b] Number of abnormal livers (includes tumors, discolorations and lesions) divided by number of livers examined.

[c] Includes only livers with tumors divided by number of livers examined.

August 19, 1974

1003536322

CTR-2 Table 3: Effect of Nitrosamines* on Liver Histology at 12 Months

Pathology	Trioc .05 ml	DMN** .05 mg	DEN .01 mg	PIP .05 mg	PYR .13 mg	DBN .40 mg
Centrilobular congestion-fatty change	1/12 8%	-	1/24 4%	11/33 33%	4/22 18%	13/33 25%
Hyalin droplet degeneration	-	-	6/24 25%	3/33 9%	4/22 18%	17/53 32%
Hepatoma	-	-	2/24 8%	-	-	-
Nodular hyperplasia	-	-	2/24 8%	1/33 3%	1/22 5%	14/53 27%
Perivascular hymphocyte accumulation	-	-	2/24 8%	1/33 3%	-	-
Lymphocyte leukemia	-	-	1/24 4%	-	1/22 5%	-
Reticular cell neoplasm	-	-	-	1/33 3%	-	3/53 6%
Lymphocytic neoplasm	-	-	-	-	-	1/53 2%

* Nitrosamines given by intraperitoneal injection to newborn C57BL/6Cum mice.
** dimethylnitrosamine (DMN) - awaiting pathology.
diethylnitrosamine (DEN)
dibutylnitrosamine (DBN)
N-nitrosopiperidine (PIP)
N-nitrosopyrrolidine (PYR)
trioctanoin (trioc)

August 19, 1974

CTR-2A: Potential Carcinogenic Effect of Nitrosamines in C57BL/6Cum Mice.

Objective:

To test the carcinogenicity of several nitrosamines in newborn C57BL/6Cum mice.

Procedure:

Intraperitoneal (IP) inoculation of newborn C57BL/6Cum mice for acute toxicity and carcinogenicity.

Progress:

1. Mice have been on test for periods that range from 42 to 52 weeks. Mortality has been greatest with dimethylnitrosamine (48%), diethylnitrosamine (46%), and dibutylnitrosamine (42%). Female mice, particularly with these substances, appear more susceptible to death than the males.

2. No tumors or other unusual findings have been observed with the mice on test. Necropsy will be performed on mice at 14 months on test.

[redacted]

CTR-2A August 19, 1974

Table 1. Survival Incidence of Mice Exposed to Nitrosamines

Material[a] Tested	Sex	# Mice Tested	# Mice Survive	Mortality	Wks. on Test
dimethylnitrosamine	♂	40	22	45%	52
.05 mg	♀	40	21	48%	52
diethylnitrosamine	♂	51	42	18%	51
.01 mg	♀	51	31	46%	51
N-nitrosopiperidine	♂	31	26	26%	51
.05 mg	♀	22	15	32%	51
N-nitrosopyrrolidine	♂	47	24	28%	50
.13 mg	♀	48	43	10%	50
dibutylnitrosamine	♂	49	33	33%	50
.40 mg	♀	48	28	42%	50
trioctanoin	♂	12	10	17%	46
.05 ml	♀	13	9	31%	46
trioctanoin	♂	10	10	0%	42
.05 ml	♀	10	10	0%	42

[a] Nitrosamines dissolved in trioctanoin and injected intraperitoneally in newborn C57BL/6Cum mice.

1003536325

CTR-3 (W-204) August 19, 1974

Induction of Squamous Cell Carcinoma in the Respiratory Tract of Mice by Intratracheal Instillation of MCA.

Objectives:

Induction of squamous cell carcinomas in mice by intratracheal instillation of MCA as described by Nettesheim for the BC3Fl mice. This study was expanded to include the parent strains of this hybrid.

Procedure:

Four mouse strains (C57BL/6Cum, C57BL/Cum, C3H/AnfCum and BC3Fl/Cum) have been treated intratracheally with gelatin vehicle or 500μg MCA in gelatin. Mice were either given 1 treatment or 3 treatments or 6 treatments at weekly intervals. The gelatin controls all received 6 treatments.

We experienced difficulty in retaining the MCA in suspension and obtained wide variations in the dose administered. To correct this problem we sonicated extensively the MCA just prior to inoculation. In light of the poor tumor induction we obtained and results presented at the recent Seattle lung carcinogenesis meeting we feel we may have actually reduced the particle size of the MCA to such a fine level that we did not obtain as many tumors as Dr. Nettesheim's group did with less sonication. Early results in additional studies indicate this may be the case.

Our method of describing time on test is different than Nettesheim's time on test. Since we treated mice 1x, 3x and 6x, we found it easier to describe time on test based on 1st treatment. Nettesheim reported his data on the basis of time after 6th treatment, therefore, there is a six week difference in time on test from that in our experiments.

Progress:

1. We lost numerous mice due to pneumonia during the inoculation period (table 1). Since few mice were lost in the gelatin vehicle control group it was concluded that the corrosive nature and possibly the immunosuppressive effects of MCA may have contributed to the high incidence of pneumonia. The number of animals dying during the injection period increased in proportion to the number of injections. With 1 instillation 2-22% died, with 3 treatments 30-47% died and with 6 treatments 45-94% died. The C57BL/Cum was the most susceptible while the other strains presented approximately similar pictures. (see table 1)

Some of this pneumonia was due to Sendai as demonstrated in table 2. We apparently introduced Sendai into the lab with the groups D,E & F. These results would indicate the C3H/f was the most resistant.

2. Mice were test sacrificed at varying intervals after inoculation for gross and histological examination. This is reflected by the number of paths taken between the 7th and 30th weeks in table 3. Most mice have now been on test in excess of 70 weeks, therefore we are in the process of terminating the experiment. The results so far indicate that 1 treatment is not sufficient and 6 treatments on a weekly basis leads to too many early deaths. The number of squamous cell carcinomas has been disappointingly low. It would appear however that more squamous cell carcinomas have occurred in the C3H than the other strains. The highest incidence of other lung tumors has been in the BC3F1. The highest number of BA lesions have occurred in the C3H/f and the C57BL mice. Any definitive differences will have to wait until all mice have been sacrificed and histopathological diagnoses made.

3. Tumors and BA lesions have been transplanted into new born mice. We have had successful transplants of squamous carcinomas, keratinized BA lesions, BA lesions with alveolar adenomas in the same lung, and alveolar adenocarcinomas. These studies are still in progress. See tables 4 and 5.

Conclusions:

1. This experiment has not provided the tumor incidence expected based on Nettesheim's report, however it has provided experience in this technique of inoculation.

2. We have also concluded that the particle size of the MCA is a significant factor and future experiments will be performed without extensive sonication of the carcinogen.

3. The dose level of 500μg at weekly intervals is too great and both dose levels and intervals between injections are being investigated.

4. Familiarity with the various lung tumors and lesions has been obtained by our pathologist, Dr. B. Sass. He has collaborated with Dr. Robert M. Kovatch of San Francisco, Dr. Stewart of NCI and Mr. William Blair of Chicago and they are in agreement as to the histopathological diagnoses. Dr. Sass is at present attempting to improve his staining technique to provide better diagnosis.

W-204, CTR-3

Table 1. Intratracheal Inoculation of mice with MCA. Losses during treatment period i.e., 1x,3x,6x MCA or gelatin.

Mouse Strain	Group #	Treatment	Date Initiated (1973)	Initial # mice	Losses During Treatment Weeks Observation 1x 0	2x 1	3x 2	4x 3	5x 4	6x 5	6	# mice after final Rx
C3H/Anf ♂	IA	gel 6x	3/19	27				1				26
	IB	500 MCA 1x	3/26	48	4							44
	IC	500 MCA 3x	3/22	40	2	8	4					26
	IE	500 MCA 3x	4/27	50	1	5	12					32
	IF	500 MCA 3x	6/4	65		2	1					62
	ID	500 MCA 6x	3/20	60		5	5		3	3		44
C57BL/6 Cum ♂	IIA	gel 6x	3/20	30			2		2			26
	IIB	500 MCA 1x	3/30	51								51
	IIC	500 MCA 3x	3/22	45		2	3					40
	IIE	500 MCA 3x	4/12	50			6					44
	IIF	500 MCA 3x	5/7	61		6	16					39
	IID	500 MCA 6x	3/21	57		1	1	4	4	2		45
BC3F1/Cum ♂	IIIA	gel 6x	3/19	30					1			29
	IIIB	500 MCA 1x	3/30	50	1							49
	IIIC	500 MCA 3x	3/22	35	2	8	3					22
	IIIE	500 MCA 3x	4/11	55	1	4	8					42
	IIIF	500 MCA 3x	5/8	62			4					58
	IIID	500 MCA 6x	3/20	64		6	8	7	16	2		25
C57BL/Cum ♀	IVA	gel 6x	4/16	30				2	1	5		22
	IVB	500 MCA 1x	4/19	50								50
	IVC	500 MCA 3x	4/19	50		2	4					44
	IVE	500 MCA 3x	4/27	80		3	35					42
	IVF	500 MCA 3x	5/8	66			1					65
	IVD	500 MCA 6x	4/18	50		3	3	6	6	8		24

1003536328

CTR-3 (W-204) Table 2. Presence of Sendai Antibody* in sera taken at monthly intervals and at the time mice were sacrificed for histopathology.

		Mouse Strain							
		C3H/Anf		C57BL/6		BC3F1		C57BL	
Group #	Treatment	P/T**	%	P/T	%	P/T	%	P/T	%
A	6x gelatin	0/15	0	1/16	6	0/17	0	1/18	6
B	1x MCA	0/27	0	0/30	0	0/30	0	0/30	0
C	3x MCA	0/15	0	0/24	0	0/10	0	0/30	0
E	3x MCA	2/17	12	0/28	0	32/37	86	19/36	53
F	3x MCA	0/28	0	17/25	68	13/42	31	13/43	30
D	6x MCA	2/29	7	9/27	33	0/16	0	11/18	61

* Sendai antibody was detected primarily during the 1st and second month the mice were on test.

** # positive mice/total number tested.

These results would indicate that the initial experimental animals in groups A, B, C, D were generally less affected. The exception was the C57BL/6 & the C57BL group D's and was due to the extended treatment period which overlapped the treatment periods of groups E and F. The spread of Sendai was limited since we isolated the sick animals as quickly as possible.

9/25/73

1003536329

CTR-3, (W-204)

Table 3. Histopathology on Lungs from Mice Treated Intratracheally with Gelatin on 500μg MCA in Gelatin at 10 Weeks of Age.

Mouse Strain / Histopathology	6x Gel 7-20	21-30	51-60	61-70	T	1x MCA 7-20	21-30	31-40	41-50	51-60	61-70	71-80	T
C3H/AnfCum													
# Paths	4	0	5	5	14	0	0	0	0	7	1	0	8
Negative	3		3	5	11					3	1		4
Pneumonia	1		0	0	1					0	0		0
BA Lesions	0		1	0	1					0	0		0
Tumors	0		0	0	0					3	1		4
Sq. c.c.	0		0	0	0					0	0		0
C57BL/6Cum													
# Paths	2	0	0	0	2	4	4	2	0	5	0	0	15
Negative	2				2	4	2	2		0			8
Pneumonia	0				0	0	0	0		1			1
BA Lesions	0				0	0	0	0		1			1
Tumors	0				0	0	2	0		2			4
Sq. c.c.	0				0	0	0	0		0			0
C57BL/Cum													
# Paths	13	0	2	0	15	6	0	1	8	2	0	0	17
Negative	13		1		14	6		1	0	1			8
Pneumonia	0		0		0	0		0	0	1			1
BA Lesions	0		1		1	0		0	2	0			2
Tumors	0		0		0	0		0	2	0			2
Sq. c.c.	0		0		0	0		0	0	0			0
BC3F1/Cum													
# Paths	0	3	1	0	4	0	0	0	0	5	0	0	5
Negative		1	1		2					1			1
Pneumonia		1	1		2					1			1
BA Lesions		1	0		1					2			2
Tumors		0	0		0					4			4
Sq. c.c.		0	0		0					0			0

Paths = Number of Pathologies taken
Sq. c.c.= Squamous cell carcinomas
B.A. = Broncho-alveolar lesions
T = Total

CTR-3, (W-204)

Contd.- Table 3. Histopathology on Lungs from Mice Treated Intratracheally with Gelatin on 500µg MCA in Gelatin at 10 Weeks of Age.

Mouse Strain Histopathology	3x MCA 7-20	21-30	31-40	41-50	51-60	61-70	71-80	T	6x MCA 7-20	21-30	31-40	41-50	51-60	61-70	71-80	T
C3H/AnfCum																
# Paths	21	24	1	16	6	0	0	66	13	14	2	0	7	7	6	43
Negative	13	16	0	0	1			30	11	5	1		0	0	0	17
Pneumonia	0	1	0	2	0			3	0	0	0		2	1	0	3
BA Lesions	8	3	1	3	1			16	1	5	0		3	6	0	15
Tumors	1	4	1	12	4			20	0	4	1		6	7	0	18
Sq. c.c.	4	1	1	2	1			8	1	0	1		2	0	0	4
C57BL/6Cum																
# Paths	21	25	8	11	2	1	0	68	18	12	0	3	1	0	0	34
Negative	16	15	3	1	0	0		35	11	6		0				17
Pneumonia	3	4	3	3	2	1		16	3	2		1	1			7
BA Lesions	3	6	3	6	1	0		19	3	3		2	1			9
Tumors	1	2	3	5	2	1		14	2	2		1	1			6
Sq. c.c.	0	0	3	2	0	0		5	0	0		1	0			1
C57BL/Cum																
# Paths	57	20	4	24	2	0	0	107	26	0	0	1	1	1	0	29
Negative	32	11	2	0	1			46	15			0	1	1		17
Pneumonia	8	3	0	6	0			17	8			0	0	0		8
BA Lesions	12	6	1	19	1			39	2			1	0	0		3
Tumors	4	0	0	13	0			17	0			1	0	0		1
Sq. c.c.	1	0	1	2	0			4	2			0	0	0		2
BC3F1/Cum																
# Paths	13	11	0	16	14	1	0	54	12	6	0	1	5		0	24
Negative	9	6		1	0	0		16	6	2		0	0			8
Pneumonia	0	4		4	3	0		12	5	1		0	3			9
BA Lesions	3	2		9	6	0		20	1	3		0	3			7
Tumors	0	0		13	12	0		25	2	1		1	6			10
Sq. c.c.	0	1		3	4	1		8	0	0		0	0			0

Paths = Number of Pathologies taken

Sq. c.c. = Squamous cell carcinomas.

B.A. = Broncho-alveolar Lesions

T = Total

1003536334

CTR-3, (W-206,W-207,W-208,W-209)

Table 4. Successful Lung Tumor Transplants in Various Strains of Mice.

Mouse Strain	Rx	MCA (µg)	# days on test	Path Diagnosis	Days Required to Reach 1cm Transplant P1*	P2	P3
C3H/f	6x	500	382	Squamous cell carcinoma	29	30	51
C3H/f	6x	500	404	Alveolar adenoma	35	57	
C3H/f	3x	500	350	-	80		
C57BL/6	3x	500	259	BA lesions, Keratinz.	45	97	
C57BL/6	6x	500	342	Squamous cell carcinoma	23		
C57BL/6	3x	500	358	Squamous cell carcinoma	80		
C57BL/6	3x	500	365	BA lesions, Alve. adenoma	45	37	
C57BL/6	3x	500	362	Alveo. adenocarcinoma	34	55	
BC3F1	3x	500	311	Adenoma, Carcinoma	91		
BC3F1	6x	500	376	Alveo. Adenocar., pneu.	85		
BC3F1	6x	500	376	Alveolar ademona	83		
BC3F1	1x	500	386	Alve. Adenoma, Alveolar adenocarcinoma	59	53	
BC3F1	3x	500	357	Alve. adenocar., BA les.	100		
BC3F1	3x	500	384	Alve. carcinoma, Alv. adenoma	100	53	
BC3F1	3x	500	384	Alve.Adenoma, car. of pleura	13	30	57
BC3F1	3x	500	357	Sq. cell car.,Alv.aden.	43		
BC3F1	3x	500	365	Squamous metastasis	35	57	
BC3F1	3x	500	397	-	87		
C57BL	3x	500	304	Papill. scirrhus, Sq. cell carcinoma	59	37	

* P = Passage #

CTR-3, (W-206)

Table 5. Unsuccessful Lung Tumor Transplants Held for Four Months

Mouse Strain	Rx	MCA (µg)	# Days on Test	Path Diagnosis
C3H/f	3x	500	284	BA Lesions
C3H/f	3x	500	284	BA Lesions, Fibrosis
C3H/f	6x	Gel	371	Essentially normal
C3H/f	6x	Gel	371	Hyperplasia of spleen
C3H/f	6x	Gel	371	1 mild BA lesion
C3H/f	6x	Gel	371	Essentially normal
C3H/f	6x	Gel	371	Essentially normal
C3H/f	1x	500	376	Lungs normal
C3H/f	6x	500	382	Broncho-Alveolar Adenoma, BA Lesions
C3H/f	6x	500	392	BA Lesions (mild)
C3H/f	1x	500	386	Essentially normal
C3H/f	3x	500	376	Essentially normal
C3H/f	6x	500	404	Alveolar carcinoma, bronch. epith. hyperplasia
C3H/f	6x	500	426	Path not available at this time.
C3H/f	1x	500	420	
C3H/f	6x	500	426	
C3H/f	6x	500	426	
C3H/f	6x	500	426	
C3H/f	3x	500	350	

CTR-3, (W-207, W-208)

Contd.- Table 5. Unsuccessful Lung Tumor Transplants Held for Four Months.

Mouse Strain	Rx	MCA (µg)	# Days on Test	Path Diagnosis
C57BL/6	6x	500	342	BA Lesions
C57BL/6	1x	500	357	Normal
C57BL/6	1x	500	363	Essentially normal
C57BL/6	1x	500	363	Interstitial pneumonia
C57BL/6	3x	500	337	Severe BA Lesions
C57BL/6	1x	500	362	Mild BA Lesions
BC3F1	1x	500	372	Alveolar adenoma, adenocarcinoma
BC3F1	1x	500	372	Alveolar adenoma
BC3F1	6x	500	382	Alveolar adenocarcinoma
BC3F1	3x	500	333	Sq. cell carcinoma, Alveolar adenoma
BC3F1	3x	500	370	Moderate BA Lesions
BC3F1	3x	500	370	Alveolar adenoma, BA Lesions
BC3F1	3x	500	343	Essentially normal
BC3F1	3x	500	384	Alveolar Adenoma, BA Lesions
BC3F1	3x	500	357	Interstitial pneu., broncho-adenoma
BC3F1	1x	500	396	Essentially normal
BC3F1	6x	500	414	Alveolar adenocarcinoma, BA Lesions
BC3F1	6x	Gel	415	Essentially normal
BC3F1	3x	500	365	Adenocarcinoma

CTR-3, (W-208, W-209)

Contd - Table 5. Unsuccessful Lung Tumor Transplants Held for Four Months.

Mouse Strain	Rx	MCA (μg)	# Days on Test	Path Diagnosis
C57BL	3x	500	337	Alveolar Adenoma, BA Lesions
C57BL	3x	500	318	Alveolar Adenoma, BA Lesions
C57BL	6x	Gel	345	Normal
C57BL	6x	Gel	345	Moderate BA Lesions
C57BL	1x	500	342	BA Lesions, Alveolar Adenoma
C57BL	1x	500	342	Essentially normal
C57BL	1x	500	342	1 Alveolar Adenoma
C57BL	1x	500	342	Essentially normal
C57BL	1x	500	342	Mild BA lesions
C57BL	1x	500	342	Essentially normal
C57BL	1x	500	342	Essentially normal
C57BL	1x	500	342	Essentially normal
C57BL	3x	500	343	Alveolar Adenoma, BA Lesions
C57BL	3x	500	343	Squamous cell carcinoma
C57BL	3x	500	343	Alveolar Adenoma, BA Lesions, pneumonia
C57BL	6x	500	344	Alveolar adenoma, BA Lesions
C57BL	3x	500	343	Squamous cell carcinoma
C57BL	3x	500	336	BA lesions
C57BL.	3x	500	336	BA lesions

1003536335

CTR-3A (CW048) August 19, 1974

Induction of Squamous Cell Carcinoma in the Respiratory Tract of Mice by Intratracheal Instillation of MCA.

Objectives:

Further studies with MCA intratracheal instillation for the induction of squamous cell carcinomas. These studies have compared the use of gelatin and trioctanoin as the vehicle and the use of treatment schedules of once a week vs. once every two weeks.

Procedure:

C3H/fMai, male and female mice have been given either 500µg MCA in gelatin or 250µg MCA in trioctanoin, once a week or once every two weeks. Our original intention was to give 500µg in each diluent, however MCA was not soluble at this level in the .02ml used for IT inoculations.

Progress:

1. These studies with the MCA given weekly were terminated at 15 weeks while the rest of the study is in the 33rd week after the initial dose of MCA. The survival rate, during the treatment period, of mice receiving 500µg MCA in gelatin at weekly intervals was only 9-36%. When MCA in gelatin was given every other week 43-48% of the mice survived. Mice treated with 250µg MCA in trioctanoin survived significantly better with almost as many control mice dying as the MCA treated mice. (see table 1)

2. The mice receiving MCA in gelatin at biweekly intervals have died or had to be sacrificed at a significantly greater rate than those receiving MCA in trioctanoin. Histological examination of five of these mice has demonstrated that all had squamous cell carcinomas. See table 2.

Comments:

This experiment very quickly demonstrates that possibly the dose of MCA given was toxic and that possibly survival rate might be improved by lower doses at 2 week intervals. Early histological evidence indicates that squamous cell carcinomas can be produced in approximately 30 weeks after the initial 500µg MCA instillation in gelatin on a 1x/week basis.

1003536336

CTR-3A Table 1

Effects of schedule, vehicle, and dose of MCA given intratracheally on survival of C3H/f male and female mice

	Treatment		Sex	Number of mice on test		33 wk. survival results	
μg MCA	Vehicle	Schedule		# of mice after 6Rx/ initial # of mice	% Surv.	# of mice surv./ # of mice on test	% Surv.
0	gelatin	1x/week	♂	24/25	96%	--- Terminated	
			♀	21/25	84	--- at 17 weeks	
500	gelatin	1x/week	♂	7/75	9	--- due to few	
			♀	9/25	36	--- surviving mice	
0	gelatin	1x/2 wks	♂	24/25	92	23/23.	100%
			♀	24/25	96	24/24	100
500	gelatin	1x/2 wks	♂	32/75	43	3/32	9
			♀	12/25	48	12/12 at 24 wks.	100
0	trioc.	1x/week	♂	24/25	96	23/24	96
			♀	19/25	76	18/19	95
250	trioc.	1x/week	♂	54/75	72	49/54	91
			♀	20/25	80	12/20	60
0	trioc.	1x/2 wks	♂	10/25	40	9/10	90
			♀	15/25	60	15/15	100
250	trioc.	1x/2 wks	♂	52/75	69	48/52	77
			♀	19/25	76	16/19	84

CTR-3A (C048) Table 2: Histopathology on Lungs from C3H/fMai Mice Treated Intratracheally with MCA in Gelatin or Trioctanoin Sex Times on a Weekly or Biweekly Schedule.

	6x (1x/week)					6x (1x/biweekly)				
	Weeks on Test				Total	Weeks on Test				Total
Dose # Paths	20-30	31-40	41-50	51-60		20-30	31-40	41-50	51-60	
Gelatin										
# Paths										
Negative										
Pneumonia										
BA Lesions										
Tumors										
Sq. c.c.										
Pleural Invas.										
Metastasis										
Trioc										
# Paths										
Negative										
Pneumonia										
BA Lesions										
Tumors										
Sq. c.c.										
Pleural Invas.										
Metastasis										
500µg MCA/Gel										
# Paths						5				5
Negative						0				0
Pneumonia						0				0
BA Lesions						3				3
Tumors						5				5
Sq. c.c.						5				5
Pleural Invas.						5				5
Metastasis						0				0
250µg MCA/Trioc										
# Paths										
Negative										
Pneumonia										
BA Lesions										
Tumors										
Sq. c.c.										
Pleural Invas.										
Metastasis										

CTR-3B (CW052) August 19, 1974

Induction of Squamous Cell Carcinoma in the Respiratory Tract of Mice by Intratracheal Instillation of MCA.

Objectives:

To repeat CTR-3A using MCA in gelatin and trioctanoin, given at weekly and biweekly intervals in C57BL/6Cum mice.

Procedure:

C57BL/6Cum male mice were used for this study since female mice were not available. 500µg MCA in gelatin or 250µg MCA in trioctanoin was given at weekly or biweekly intervals. Due to the high incidence of deaths in mice treated with 500µg MCA in gelatin at weekly intervals only 5 treatments were given. All other mice received 6 treatments.

Progress:

1. This study is in the 33rd week after the initial treatment. No evidence of respiratory difficulty is seen in the mice remaining from the weekly MCA-gel treatments while the biweekly treated mice appear sick and deaths are occurring. Mice given MCA in trioctanoin are healthy.

2. The initial deaths and the surviving mice at this time are seen in table 1.

3. Histopathological studies on 22 sick mice, 27-29 weeks after the initial inoculation (13-17 weeks after the 6th biweekly injection), have demonstrated squamous cell carcinomas in all mice. We will now start to test sacrifice mice for additional indications of tumor induction.

1003536339

CTR-3B Table 1

Effects of schedule, vehicle, and dose of MCA given intratracheally on survival of C57BL/6 mice

Treatment			Number of mice on test		33 week survival results	
µg MCA	Vehicle	Schedule	# of mice after Rx/ initial # of mice	% Surv.	# of mice surv./ # of mice on test	% Surv.
0	gelatin	6x/1x wk.	43/45	93%	42/43	98%
500	gelatin	5x/1x wk.	36/100	36	24/36	67
0	gelatin	6x/1x Biweekly	49/50	98	48/49	98
500	gelatin	6x/1x Biweekly	62/100	62	26/36	72
0	trioc.	6x/1x wk.	45/50	90	45/45	100
250	trioc.	6x/1x wk.	78/100	78	78/78	100
0	trioc.	6x/1x Biweekly	45/90	90	45/45	100
250	trioc.	6x/1x Biweekly	89/100	89	86/89	97

1003536340

CTR-3A (C052) Table 2: Histopathology on Lungs from C57BL/6 Mice Treated Intratracheally with MCA in Gelatin or Trioctanoin 6 Times on a Weekly or Biweekly Schedule.

	6x (1x/week)					6x (1x/biweekly)				
	Weeks on Test				Total	Weeks on Test				Total
Dose # Paths	20-30	31-40	41-50	51-60		20-30	31-40	41-50	51-60	
Gel										
# Paths										
Negative										
Pneumonia										
BA Lesions										
Tumors										
Sq. c.c.										
Pleural Invas.										
Metastasis										
Trioc										
# Paths										
Negative										
Pneumonia										
BA Lesions										
Tumors										
Sq. c.c.										
Pleural Invas.										
Metastasis										
250.0µg MCA/Trioc										
# Paths						1				1
Negative						1				1
Pneumonia						0				0
BA Lesions						0				0
Tumors						0				0
Sq. c.c.						0				0
Pleural Invas.						0				0
Metastasis						0				0
500.0µg MCA/Gel										
# Paths						21				21
Negative						0				0
Pneumonia						0				0
BA Lesions						11				11
Tumors						20				20
Sq. c.c.						21				21
Pleural Invas.						19				19
Metastasis						2				2

CTR-3C (CW055) August 19, 1974

Induction of Squamous Cell Carcinoma in the Respiratory Tract of Mice by Intratracheal Instillation of MCA.

Objectives:

Based on the two previous studies with MCA intratracheal injection it would appear that smaller doses must be used to allow the mice to survive the inoculation period; therefore this experiment was undertaken to determine if tumor induction could be obtained with minimal doses.

Procedure:

C3H/f female mice were injected with either 62.5, 125 or 250 µg MCA in gelatin at weekly intervals for 6 and 12 times.

Progress:

1. The mice in this study are now on for 28 weeks after the initial MCA treatment.

2. The deaths through these treatment schedules and during the following observation period have significantly been reduced. (see table 1)

3. Seven mice have been autopsied 21-23 weeks after initial MCA injection (total of 12 treatments) and all were found to have squamous cell carcinomas. (see table 2)

CTR-3C Table 1

Effects of dose and number of times of IT administered on the survival of C3H female mice

Number of treatments	MCA dose (μgm)	Number of mice on test: # of mice after final Rx/initial # of mice	% Surv.	28 week survival results: # of mice dead/ # of mice on test	% Surv.
6	0	24/25	96	24/24	100
	62.5	47/50	94	42/47	89
	125.0	49/50	98	48/49	98
	250.0	44/50	88	34/44	77
12	62.5	40/47	85	37/40	93
	125.0	38/46	83	33/38	87
	250.0	31/51	61	24/31	77

CTR-3C (C055) Table 2: Histopathology on Lungs from C3H/f Female Mice Treated Intratracheally 6-12 Times with Three Dose Levels of MCA, One Time/Week.

Dose # Paths	6x (1x/week) Weeks on Test 20-30	31-40	41-50	51-60	Total	12x (1x/week) Weeks on Test 20-30	31-40	41-50	51-60	Total
Gelatin										
# Paths										
Negative										
Pneumonia										
BA Lesions										
Tumors										
Sq. c.c.										
Pleural Invas.										
Metastasis										
62.5µg MCA										
# Paths	1				1	1				1
Negative	1				1	0				0
Pneumonia	0				0	1				1
BA Lesions	0				0	0				0
Tumors	0				0	0				0
Sq. c.c.	0				0	0				0
Pleural Invas.	0				0	0				0
Metastasis	0				0	0				0
125.0µg MCA										
# Paths						3				3
Negative						0				0
Pneumonia						0				0
BA Lesions						2				2
Tumors						2				2
Sq. c.c.						2				2
Pleural Invas.						1				1
Metastasis						0				0
250.0µg MCA										
# Paths	1				1	8				8
Negative	0				0	0				0
Pneumonia	0				0	0				0
BA Lesions	0				0	6				6
Tumors	1				1	6				6
Sq. c.c.	1				1	7				7
Pleural Invas.	0				0	2				2
Metastasis	0				0	[illegible]				1

32A.

1003536344

CTR-3D August 19, 1974

Induction of Squamous Cell Carcinoma in the Respiratory Tract of Mice by Intratracheal Instillation of MCA.

Objectives:

To repeat CTR-3C in male mice to determine if there is a difference in susceptibility between male and female mice.

Procedure:

C3H/f male mice will be inoculated 6-12 times with varying doses of MCA via IT route at weekly intervals.

Progress:

This experiment was initiated August 12, 1974.

1003536345

CTR-4 (W-200) August 14, 1974

Subcutaneous Treatment of C57BL, C3H/Anf and BC3F1 Mice with 3-Methylcholanthrene for Comparison with Intratracheal Treatment for Tumor Induction.

Objectives:

Several mouse strains were selected in CTR-3 for lung carcinogenesis studies which had not previously been studied in this laboratory by subcutaneous MCA treatment. In order to establish their relative susceptibility with previously tested mouse strains by the same criteria, studies were undertaken using our standard subcutaneous evaluation procedures.

Procedure:

Weanling mice of seven strains were given subcutaneous injections of three doses of MCA to determine relative susceptibility to subcutaneous tumor induction.

Progress:

This study has been completed with the establishment of relative susceptility to MCA given subcutaneously, the AHH inducibility and the presence of group specific (gs) antigens for the type C RNA tumor viruses. (see tables)

Conclusions:

1. The C3H/f mice are the most susceptible of the strains tested to MCA subcutaneous tumorigenesis (table 1). There is probably no significant difference in the C3H/f mice from Microbiological Associates and Cumberland View Farms, although some differences were noted with 37.5μg MCA. The Mai strain has consistently given almost as many tumors with 37.5μg and 150μg. This was the first time we had run three doses with the Cum strain. One of the most significant factors in the high degree of susceptibility of this strain is the short latency period.

2. The C57BL and the C57BL/6 do not show significant differences in MCA tumorigenicity (table 1). This was our first experience with the parent strain - the C57BL.

3. The hybrid mice strains (BC3F1/Cum and B6C3F1/Mai) do not differ significantly in tumor indicence, however the latency is greater in the B6C3F1/Mai mice. We did not test the C57BL/6Mai mice in this study, however previous studies have demonstrated significant lower susceptibility to subcutaneous MCA carcinogenesis in the hybrids than the C57BL/6Cum strain. The Mai strain also has higher levels of gs antigen than the Cum strain. One of the significant findings of this study is the apparent in- increased susceptibility of the hybrid when the B6/Cum was crossed with the C3/Mai and the susceptibility approached

1003536346

that of the C3H parent. This strain is not commercially available. This strain should be characterized for susceptibility to IT inoculation with a variety of carcinogens.

4. Spleens and tumors have been tested by CF test for gs antigen to determine type C RNA viral genome expression. As seen in Table 2 the C57BL/6 and C57BL mice were virtually negative for gs antigen as previously observed. The C3H/Anf and C3H/f were low in gs antigen at 4 weeks, however, the MCA induced tumors were nearly all positive even at 1:8 dilutions of the 40% tumor sonicate extract. The BC3F1 appeared to take on the same gs antigen expression as the C3H/Anf parent which reflects previous findings with the C57BL/6Mai and C3H/fMai parents. The Mai strain of C57BL/6 has more gs antigen expression than the C57BL/6Cum strain. The B6C3F1/Cum x Mai mice were bred in our laboratory from C57BL/6Cum females and C3H/fMai males. It would appear from the control mice sacrificed at 4 weeks of age to have comparable gs antigen expression as the B6C3F1/Mai, therefore, the C3H/fMai may have contributed the gs antigen expression tendency to the hybrid strains.

5. The AHH inducibility (table 3) of the C57BL, C57BL/6 and BC3F1 mice are all similar and are more inducible than the C3H mice. The significance of differences in the degree of inducibility is not known. It is felt, however, that if an animal is inducible, the initial event of transformation occurs which governs the tumor incidence. The latency of tumor development is probably not dependent on inducibility but rather on other host related factors, as immunocompetence.

6. The BC3F1 mice were also used in CTR-3 and found to be a significantly more sturdy strain than either of the parents. The hybrid has the nice features of the C3H/f in that it is easy to handle, does not fight nor develop skin lesions common to the C57BL/6. The BC3F1 exhibits the barbering character common to approximately 10% of the C57BL parents, however in the BC3F1 barbering occurs in virtually 100% of the mice and is confined to the nose and eye region. In the C57BL/6 mice they also barber the shoulder region.

Comments:

The C57BL/6 strain produces few tumors with DMBA or BP while the C3H/f is highly susceptible. As a further characterization of the hybrid strain these studies have been included in a later study (CTR-19).

CTR-4, (W-200) -- Table 1: Subcutaneous Carcinogenic Effects of MCA in Various Strains of Mice (8 Months Observation)

Mouse Strain	MCA Dose (µg)	Tu/T	%	Latency (wks) Av.	50%	Range	CI	TD_{50} µg MCA
C3H/AnfCum	9.38	9/29	31	20.7	23.2	18-35	21	13
	37.5	16/29	55	17.1	13.8	11-35	46	
	150.0	25/29	86	13.9	10.2	8-23	89	
C3H/fMai	9.38	9/28	32	22.6	18.0	15-35	20	20
	37.5	24/29	83	17.5	14.8	13-33	68	
	150.0	27/30	90	13.4	9.9	9-24	96	
C57BL/Cum	9.38	2/22	9	28.5	27.0	27-30	5	18
	37.5	14/25	56	22.8	20.0	16-30	35	
	150.0	18/25	72	17.5	15.0	13-23	59	
C57BL/6Cum	9.38	3/27	11	22.6	18.0	18-25	7	40
	37.5	12/28	43	21.2	19.5	12-34	29	
	150.0	19/30	63	15.8	15.1	14-27	57	
$BC3F_1$/Cum (C57BL x C3H/Anf)	9.38	3/30	10	21.3	20.5	20-23	7	82
	37.5	8/30	27	20.5	14.0	11-35	19	
	150.0	22/30	73	14.0	12.2	9-24	75	
B6C3F1/Mai (C57BL/6 x C3H/f)	9.38	0/30	0	-	-	-	-	82
	37.5	10/30	33	19.8	16.8	13-23	24	
	150.0	19/30	63	17.7	14.1	10-28	51	
B6C3F1 (Cum x Mai)	9.38	4/21	19	21.8	21.0	21-24	13	12
	37.5	19/30	63	20.5	18.6	8-29	44	
	150.0	25/30	83	15.7	11.2	9-32	75	

1003536348

CTR-4 Table 2 Comparison of Type C RNA gs antigen expression among the various strains used for subcutaneous and intratracheal MCA tumor induction.

Mouse Strain	Tissue (40%)	gs Antigen P/T* Untreated Control Mice** 1:2	1:4	1:8	MCA-Tumored Mice*** 1:2	1:4	1:8
C57BL/6Cum	Spleen	0/4			0/5		
	Tumor				1/5	1/5	0/5
C57BL/Cum	Spleen	0/5			0/5		
	Tumor				0/5		
C3H/AnfCum	Spleen	2/4	1/4	0/4	4/5	2/5	2/5
	Tumor				4/5	4/5	4/5
C3H/fMai	Spleen	1/6	1/6	0/6	1/5	1/5	0/5
	Tumor				5/5	5/5	5/5
BC3F1/Cum	Spleen	0/5			1/5	1/5	0/5
	Tumor				1/5	1/5	0/5
B6C3F1/Mai	Spleen	2/5	1/5	0/5	3/3	2/3	2/3
	Tumor				3/3	3/3	3/3
B6C3F1/Cum x Mai	Spleen	2/5	1/5	0/5	No data at this time.		
	Tumor				" " " " "		

*P/T number positive at = 3+ complement fixation/Total number specimens tested.

**Untreated mice sacrificed at 4 weeks of age at time 150μg MCA administered to test mice.

***Tumored mice were sacrificed when MCA subcutaneous tumors were 2 cm in size at 10-15 weeks after MCA treatment.

Table 3.

AHH inducibility of strains of Mice used in CTR-3 and CTR-4.

Mouse Strain	AHH Specific Activity Control	MCA-treated	Inducibility
C3H/AnfCum	4.3	18.4	4.25
C3H/fMai	3.6	14.5	3.96
$BC3F_1$/Cum	3.4	29.7	8.80
C57BL/6Cum	4.5	28.1	6.30
C57BL/Cum	4.5	31.3	7.03
B6C3F1/Cum*	6.1	33.9	5.58
$B6C3HF_1$/Mai	5.0	41.6	8.32

*C57BL/6Cum and C3H/AnfCum were bred in our laboratory.

CTR-5 August 19, 1974

Relationship Between the Sensitivity to MCA-Induced Squamous Cell Carcinomas and Inducibility of AHH Activity

Objectives:

To determine the role of AHH in carcinogenesis induced by MCA.

Procedure:

C57BL/6 and DBA/2 strains of mice have been mated to obtain F1 and backcross animals. AHH inducibility segregates as a single autosomal gene in this cross. 500μg MCA in 0.02 ml of sterile 0.2% gelatin was given IT to these mice once a week for a total of 3 or 6 weeks.

Progress:

1. The various groups, their date of initiation and relative toxicity are shown in table 1.

2. Animals from selected groups were killed and observed for pathological lesions. The results are in table 2.

3. In the next 30 days all animals will be taken off test, observed macroscopically and processed for pathology.

Conclusions:

These results agree with CTR-3, in that a very low tumor response was initially observed. Discussions with Dr. P. Nettesheim and Mr. W. Blair have indicated that the particle size of the MCA may have been the problem. Our new results (see CTR-3A, B & C) with various doses of MCA using different treatment schedules and vehicles, indicate that conditions can be made whereby viability is high and the number of animals showing early macroscopic lesions are proportionally high. No pathological diagnosis is available at this time. These conditions seem to be 1) large particle size, 2) low pneumonia incidence, 3) careful handling of the individual animals, and 4) use of older (10-12 week old) animals. This genetic experiment is being repeated in CTR-39.

CTR-5

Table I

Intratracheal Inoculation of Mice. losses During Treatment Period.

Strain		T#	Treatment	Date Initiated	Initial #Mice	Time on Test	Age on Test	Animals lost during treatment 1x	2x	3x	# Remain on Test 8/20/74
DBA/2	♂	0269	Gel 500 MCA 3x	5/10/73	60	46 wks.	8-9 wks.	10	41	9	0
				7/25/73	60	36 wks	12 wks.	1	0	39	20
DBA/2	♀	0270	Gel 500 MCA 3x	5/10/73	60	46 wks.	8-9 wks.	5	3	46	6
$B6D2F_1$	♂	0271	Gel 500 MCA 3x	5/17/73	60	45 wks.	9-10 wks.	0	0	37	23
$B6D2F_1$	♀	2072	Gel 500 MCA 3x	5/17/73	60	45 wks.	10-11 wks.	0	0	60	0
C57BL/6	♂	2076	Corn Oil 375 MCA 3x	6/13/73	65	41 wks.	8-9 wks.	0	2	41	22
C57BL/6	♀	2077	"	6/13/73	65	41 wks.	8-9 wks.	5	4	32	24
C57BL/6	♂	0286	Corn Oil 3x	6/27/73	10	39 wks.	10 wks.	0	0	5	5
C57BL/6	♀	0287	Corn Oil 3x	6/27/73	10	39 wks.	10 wks.	3	0	4	3
B6D2 D2	♂	0288	Gel 500 MCA 3x	7/26/73	9	36 wks.	7-12 wks.	0	0	8	1
				8/ 2/73	10	35 wks.	7-12 wks.	0	0	9	1
				8/10/73	4	33 wks.	7-12 wks.	0	1	3	0
				11/21/73	19	19 wks.	7-12 wks.	0	0	13	6
B6D2·D2	♀	0289	Gel 500 MCA 3x	7/26/73	8	36 wks.	7-12 wks.	0	0	4	4
				8/ 2/73	2	35 wks.	7-12 wks.	0	0	2	0
				8/10/73	7	33 wks.	7-12 wks.	0	3	4	0
				11/21/73	6	19 wks.	7-12 wks.	0	0	4	2
B6·B6D2	♂	0291	Gel 500 MCA 3x	7/26/73	26	36 wks.	7-12 wks.	0	0	15	11
				8/ 2/73	16	35 wks.	7-12 wks.	0	0	13	3
				8/10/73	3	33 wks.	7-12 wks.	0	0	3	0
				11/13/73	11	20 wks.	7-12 wks.	0	0	8	3
				11/21/73	6	19 wks.	7-12 wks.	0	0	2	4
B6·B6D2	♀	0290	Gel 500 MCA 3x	7/26/73	34	36 wks.	7-12 wks.	0	0	26	8
				8/ 2/73	21	35 wks.	7-12 wks.	0	0	20	1
				11/13/73	11	20 wks.	7-12 wks.	0	0	10	1
				11/21/73	10	19 wks.	7-12 wks.	0	0	7	3
B6D2·B6	♀	0292	Gel 500 MCA 3x	7/26/73	2	36 wks.	7-12 wks.	0	0	2	0
D2·B6D2	♂	0293	Gel 500 MCA 3x	8/ 2/73	8	35 wks.	7-12 wks.	0	0	5	3
				8/10/73	4	33 wks.	7-12 wks.	0	0	4	0
				11/21/73	5	19 wks.	7-12 wks.	0	0	1	4
	♀	0294	Gel 500 MCA 3x	8/ 2/73	5	35 wks.	7-12 wks.	0	0	3	2
				8/10/73	2	33 wks.	7-12 wks.	0	0	1	1
				11/13/73	4	20 wks.	7-12 wks.	0	0	3	1
				11/21/73	2	19 wks.	7-12 wks.	0	0	2	0
D2·D2B6	♂	0315	Gel 500 MCA 3x	11/13/73	6	20 wks.	7-12 wks.	0	0	4	2
D2·D2B6	♀	0316	Gel 500 MCA 3x	11/13/73	5	20	7-12 wks.	0	0	5	0

CTR-5 Table 2 Pathology Results

Strain	Sex	Time on Test	Rx	# of mice	Results
C57BL/6	♂	182(days)	375 MC IT (3x)	1	Endobronchial abcesses, bronchopneumonia
C57BL/6	♂	287	375 MC IT (3x)	1	Interstitial pneumonia, emphysema
C57BL/6	♂	287	375 MC IT (3x)	1	Interstitial pneumonia, erythroid hyperplasia spleen
C57BL/6	♂	287	375 MC IT (3x)	2	emphysema, pneumonia, erythroid hyperplasia spleen
C57BL/6	♂	287	375 MC IT (3x)	1	normal
C57BL/6	♂	287	375 MC IT (3x)	1	emphysema, adenoma, pneumonia, hyperplasia of spleen
C57BL/6	♂	287	375 MC IT (3x)	1	Interstitial pneumonia, hyperplasia spleen, emphysema
C57BL/6	♂	358	375 MC IT (3x)	1	pneumonitis
C57BL/6	♀	182	375 MC IT (3x)	1	pneumonitis, endobronchial abcesses
C57BL/6	♀	182	375 MC IT (3x)	1	pneumonitis, endobronchial abcesses
C57BL/6	♀	279	375 MC IT (3x)	1	pneumonitis, hyperplasia spleen
C57BL/6	♀	279	375 MC IT (3x)	1	pneumonitis, hyperplasia spleen
C57BL/6	♀	358	375 MC IT (3x)	1	pneumonitis, hyperplasia spleen
B6D2F$_1$	♂	85	500 MC IT (3x)	1	hyperplasia spleen
B6D2F$_1$	♂	85	500 MC IT (3x)	1	lung 80% solid tumor mass
B6D2F$_1$	♂	85	500 MC IT (3x)	7	normal

CTR-5 Table 2: Pathology Results (con't)

Strain	Sex	Time on test	Rx	# of mice	Results
B6D2F$_1$	♂	85	500 MC IT (3x)	1	adenoma, pneumonia
B6D2F$_1$	♂	85	500 MC IT (3x)	1	squamous cell carcinoma
B6D2F$_1$	♂	240	500 MC IT (3x)	1	pneumonia, adenoma, hyperplasia of spleen
B6D2F$_1$	♂	243	500 MC IT (3x)	1	pneumonia, adenoma, lung abcesses, hyperplasia of spleen
B6D2F$_1$	♂	314	500 MC IT (3x)	1	adenoma
B6D2F$_1$	♂	385	500 MC IT (3x)	1	pneumonia, adenomas, lymphocyte neoplasms
B6D2F$_1$	♂	385	500 MC IT (3x)	1	adenocarcinoma, adenoma, red cell neoplasms, type B
B6D2F$_1$	♂	385	500 MC IT (3x)	1	pneumonia, adenoma
B6D2F$_1$	♀	235	500 MC IT (3x)	1	squamous cell carcinoma with marked keratinization - lung infarction
B6D2F$_1$	♀	238	500 MC IT (3x)	1	squamous cell carcinoma
B6D2F$_1$	♀	238	500 MC IT (3x)	1	adenomas, adenocarcinoma with infarction
B6D2F$_1$	♀	380	500 MC IT (3x)	1	pneumonia, adenocarcinoma
B6D2F$_1$	♀	385	500 MC IT (3x)	1	fibrous pneumonia
DBA/2	♂	301	500 MC IT (3x)	1	lymphocyte neoplasm
DBA/2	♂	321	500 MC IT (3x)	1	infarction, BA lesion
DBA/2	♂	321	500 MC IT (3x)	1	normal

CTR-5 Table 2: Pathology Results (con't)

Strain	Sex	Time on test	Rx	# of mice	Results
DBA/2	♀	92	500 MC IT (3x)	4	normal
DBA/2	♀	92	500 MC IT (3x)	2	hyperplasia of spleen
DBA/2	♀	245	500 MC IT (3x)	1	normal
DBA/2	♀	377	500 MC IT (3x)	1	reticulo. cell neoplasm type A; lung, liver lymph nodes w/ giant cells
DBA/2 x D2B6	♂	203	500 MC IT (3x)	1	squamous cell carcinoma
DBA/2 x B6D2	♂	306	500 MC IT (3x)	1	pneumonitis
B6D2 x DBA/2	♀	313	500 MC IT (3x)	1	pneumonitis
B6 x B6D2	♂	41	500 MC IT (3x)	1	lymphoid leukemia
B6 x B6D2	♂	41	500 MC IT (3x)	1	BA lesions, pneumonia
B6 x B6D2	♂	41	500 MC IT (3x)	1	hyperplasia of spleen
B6 x B6D2	♂	41	500 MC IT (3x)	1	pneumonia

CTR-15,16,17 August 20,1974

Effects of TCDD on MCA-Induced Tumor Formation.

Objectives:

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a potent inducer of AHH activity in various mouse strains. A dose of 100nMoles will even induce the MCA-nonresponsive strain, DBA/2. Thus, the DBA/2 strain, possesses the structural genes required for induced AHH activity and lacks (or has a defective) "recognition" protein. A dose of 1nMole TCDD will not induce the DBA/2 strain but will induce the C57BL/6 (our prototype MCA-inducible strain). The susceptiblity of these two strains to MCA carcinogenesis seems to be genetically linked to their ability to be AHH induced with MCA. This study is designed to determine if artificially induced high levels of AHH (induced by TCDD) will alter the susceptibility of either AHH-inducible (B6) or AHH-noninducible (D2) mice.

Progress:

Tables 1 and 2 show the 8 treatment groups for the DBA/2 and C57BL/6 strains, and the weekly tumor incidences for two experiments put on test on separate days. The relative toxicity at 28 days post treatment is also given. The C57BL/6 mice were very sensitive to MCA-induced tumors and no real effect of prior or simultaneous treatment with TCDD was observed (Table 1). The DBA/2 mice were relatively resistent to MCA carcinogenesis and only three treatment schedules yielded tumor incidences of any consequence. Simultaneous treatment with TCDD (especially at 100nMoles) and pretreatment (48 hrs) with the TCDD vehicle, dioxane, enhanced MCA tumorigenesis.

Conclusions:

The results with the TCDD are compatible with the idea that AHH induction (via TCDD) simultaneous with MCA treatment yields more tumors than MCA alone, but the results with dioxane are difficult to assess. Why a 48 hr. pretreatment with 0.010 ml dioxane should enhance MCA-induced tumorigenesis cannot be explained at this time. The fact that both the low and high TCDD levels, when given 48 hrs. before MCA, had no effect, yet dioxane was also in these treatments, indicates that whatever the effect of dioxane, it is cancelled, if TCDD is present. We feel that a repeat of this experiment must be done. The protocol for this repeat is CTR-40.

CTR-15, 16, 17- Effects of TCDD on MCA-induced tumors
(Combined results for mice put on test Oct. 13 & Nov. 9, 1973)

Strain	Treatment -2 days	Treatment 0 day	Toxicity[a] #	Toxicity[a] %	Tu/T[b]	%	Avg. Latency	CI[c]
DBA/2	diox	Trioc	18/40	45	0/22	0	-	-
DBA/2	TCDD(H)	Trioc	35/60	58	0/25	0	-	-
DBA/2	none	150 MCA	15/50	30	1/34	3	217	1
DBA/2	diox	150 MCA	12/40	30	6/25	24	172	14
DBA/2	TCDD(L) +150 MCA	none	11/45	24	5/34	15	199	7
DBA/2	TCDD(H) +150 MCA	none	66/110	60	10/43	23	178	13
DBA/2	TCDD(L)	150 MCA	14/45	31	0/31	0	-	-
DBA/2	TCDD(H)	150 MCA	32/60	53	0/28	0	-	-
C57BL/6	diox	Trioc	1/40	3	0/39	0	-	-
C57BL/6	TCDD(H)	Trioc	33/60	55	0/27	0	-	-
C57BL/6	none	150 MCA	4/40	10	29/36	81	125	65
C57BL/6	diox	150 MCA	7/40	18	24/31	77	119	65
C57BL/6	TCDD(L) +150 MCA	none	18/45	40	27/27	100	132	76
C57BL/6	TCDD(H) +150 MCA	none	37/80	46	33/43	77	123	63
C57BL/6	TCDD(L)	150 MCA	20/45	44	16/23	70	140	50
C57BL/6	TCDD(H)	150 MCA	35/60	58	21/25	84	129	65

[a] Toxicity given in terms of # of mice dead in 28 days.

[b] No. of tumored animals per no. of treated animals 36 weeks after treatment.

[c] Carcinogenic index

1003536357

August 19, 1974

CTR-18: Nitrosamine Induced Respiratory Tumors in DBA/2J Mice.

Objectives:

a. To establish toxicity levels for nitrosamines when instilled intratracheally at one or two week intervals several times.

b. To then evaluate the carcinogenicity of these substances in the respiratory tract of DBA/2J mice.

Procedure:

a. The nitrosamines were dissolved in corn oil to give stock solution of 25, 50 and 100 μg/.02 ml of vehicle for each treatment.

b. Groups of mice (50 to 100 each) were intratracheally (I.T.) instilled with three concentrations (25,50 and 100μg) diethylnitrosamine (DEN) at one or two week intervals. Control mice receive 0.02 ml of corn oil alone at each instillation.

Progress:

1. Preliminary trials indicated that 25, 50 and 100μg doses of DEN were not toxic to mice after IT instillation. We therefore initiated our study with chronic weekly doses of 1000 and 2000 μg of DEN per mouse.

2. The early toxic effects after 39 days on test and five treatments were shown in the December report. The higher dosage of DEN (2000μg) was about 5 times more toxic than the 1000μg dosage.

3. Mice received a total of 6 intratracheal (I.T.) injections of DEN and have currently been on test about 10 months (see attached Table 1.). Mice will be held on test and scheduled for gross and histological observation at a later date.

1003536358

CTR-18
D100

August 19, 1974

Table 1. Survival Incidence Eight Months After Nitrosamine Exposure[a].

Substance Tested	Sex	No. Weeks on Test	Mortality Dead/Tested	(%)	No. surviving Mice
diethyl-nitrosamine 1000µg	♂	43	10/50	20%	40
	♀	"	6/50	12%	44
	Both	"	16/100	16%	84
2000µg	♂	42	6/20	30%	14
	♀	"	11/20	55%	9
	Both	"	17/40	42%	23
Corn Oil .02 ml	♂	42	4/20	20%	16
	♀	"	1/20	5%	19
	Both	"	5/40	12%	35

[a]Mice received 6 intratracheal instillations at weekly intervals.

1003536359

[redacted]

CTR-18A

August 19, 1974

Nitrosamine Induced Respiratory Tumors in Mice.

Objectives:

To determine the possibility of producing lung tumors with DMN using the wax pellet procedure of Stanton. Dimethylnitrosamine (DMN) and several other nitrosamines are present in small amounts in cigarette smoke. Levels of hydrocarbon hydroxylase activity seem to play a role in the mechanism of DMN carcinogenesis for treatment with polycyclic aromatic hydrocarbon (e.g. MCA) will depress the metabolic activity of DMN by depressing levels of DMN-dimethylase. Therefore, strains most sensitive to MCA (because AHH inducible) may be very resistant to DMN tumorigenesis and vice versa. For this reason both AHH inducible and non-inducible strains have been included.

Procedure:

Since nitrosamines are very volatile the use of IT inoculation procedures as suggested in the initial proposed study was not used since it was felt there was too much danger to the technician. We have substituted the wax pellet technique.

Progress:

Preliminary studies with DBA/2 mice established very quickly that the dose of 1 mg and 0.5 mg was too toxic. We have had some deaths in the control mice due to the nature of the technique. See table 1 for progress in initiating the experiment and the deaths which occurred due to toxicity.

CTR-18A, (C-061)

Aug. 14, 1974

Table 1 - DMN Lung Implants in ♀ Mice

Mouse Strain	Treatment	Group	Date on Test	Days on Test	Weeks on Test	# Animals Placed on Test	Death[a]	Tu/T
DBA/2 J	No Carcinogen	01A1A	7/9/74	37	6	30	0	0/30
DBA/2 J	.05ml B:T	01B2A	6/27/74	48	7	30	1	0/29
DBA/2 J	1mg DMN/.05ml B:T	01C4A	6/27/74	48	7	40	40	
DBA/2 J	.5mg DMN/.05ml B:T	01C3A	6/27/74	48	7	40	40	
DBA/2 J	.25mg DMN/.05ml B:T	01C6A	8/2/74	12	2	40	6	0/34
DBA/2 J	.125mg DMN/.05ml B:T	01C5A	8/2/74	12	2	40	4	0/36
SWR/J	No Carcinogen	02A1A	7/23/74	22	4	25	1	0/24
SWR/J	.05ml B:T	02B2A	7/3/74	42	6	30	12	0/18
SWR/J	.25mg DMN/.05ml B:T	02C6A	7/3/74	42	6	40	12	0/28
SWR/J	.125mg DMN/.05ml B:T	02C5A	7/3/74	42	6	40	12	0/28
C57BL/6 Cum	No Carcinogen	03A1A	7/12/74	33	5	30	0	0/30
C57BL/6 Cum	.05ml B:T	03B2A	7/12/74	33	5	30	10	0/20
C57BL/6 Cum	.25mg DMN/.05ml B:T	03C6A	7/16/74	29	5	40	13	0/27
C57BL/6 Cum	.125mg DMN/.05ml B:T	03C5A	7/16/74	29	5	40	7	0/33
BALB/c Mai	No Carcinogen	04A1A	Scheduled					
BALB/c Mai	.05ml B:T	04B2A	for					
BALB/c Mai	.25mg DMN/.05ml B:T	04C6A	Sept. 1974					
BALB/c Mai	.125mg DMN/.05ml B:T	04C5A						

Continued on Page 2.

CTR-18A, (C-061)

Aug.14, 1974

Table 1 - DMN Lung Implants in ♀ Mice (cont.)

Mouse Strain	Treatment	Group	Date on Test	Days on Test	Weeks on Test	# Animals Placed on Test	Death[a]	Tu/T
C3H/f Mai	No Carcinogen	05A1A	7/12/74	33	5	30	0	0/30
C3H/f Mai	.05ml B:T	05B2A	7/12/74	33	5	30	4	0/26
C3H/f Mai	.25mg DMN	05C5A	7/16/74	29	5	40	11	0/29
C3H/f Mai	.125mg DMN	05C5A	7/16/74	29	5	40	5	0/35

[a]Number of animals dead due to treatment, toxicity or other.

1003536362

1003536363

Proposal 1975

A. EXPERIMENT CTR-38 : Effects of Vitamin A on lung carcinogenesis (Substitute for Supplementary study suggested as CTR-32)

1. Purpose: To determine the effects of vitamin A on pulmonary tumorigenesis, AHH inducibility and immunocompetence.

2. Background: Vitamin A is necessary to maintain normal differentiation and function of secretory epithelium. Extensive epithelial hyperplasia associated with anaplasia or squamous metaplasia has been demonstrated in tissue culture by MCA and is similar to that seen in vitamin A deficiency. Both these conditions in tissue culture can be reversed by addition of vitamin A. In hamsters, vitamin A has been shown to inhibit lung tumors by BP as well as forestomach and cervix tumor induction by DMBA or BP. DMBA and BP induced epidemoid tumors in mice and rabbits have been inhibited by retinoic acid.

The mechanism of antitumor effects of vitamin A in animals remains to be defined. There appear to be several areas where vitamin A may play a role in the defense mechanisms of the host to tumorigenesis: (1) The ability of the animal to maintain growth and cellular differentiation.(2) The ability of the microsomal bound enzyme, important in the metabolism of polycyclic aromatic hydrocarbon and nitrosamine carcinogens, to function. Protein- and protein-choline-deficient diets have been shown to influence the enzymatic functions of the cells. (3) The influence of the immune response of an animal by influencing the reticuloendothelial system. Vitamin A has been shown to prevent thymic involvement due to stress and has been important in decreasing the severity of viral infection and tumors of viral origin. (4) The influence of vitamin A on the promoting of mucopolysaccharide biosynthesis or in strengthening of extracellular barriers to chemical and viral involvement.

It is the purpose of these experiments to determine the possibility of increasing susceptibility of mice to lung carcinogenesis by chemical carcinogens. For the present studies we have selected two mouse strains, the C3H/f which is AHH inducible and highly sensitive to subcutaneous carcinogenesis and the DBA/2 which is AHH non-inducible and relatively insusceptible to PAH carcinogenesis. The initial studies will be done with MCA and BP, however, it may prove useful to investigate the significance of vitamin A in nitrosamine and tobacco smoke carcinogenesis based on these initial studies. If we can increase susceptibility to tumor induction by the use of a vitamine A deficient diet we could probably increase our chances of success in the development of mouse inhalation animal model.

3. Materials:

a. Mice
   (1) C3H/f
   (2) DBA/2

b. Chemical Carcinogens
(1) 0.2% gelatin vehicle
(2) 250µg MCA/0.02 ml 0.2% gelatin
(3) 0.6 mg Fe_2O_3
(4) 1.8 mg BP
(5) 0.6 mg Fe_2O_3 + 1.8 mg BP

c. Mouse Food
(1) Vitamin A-free diet
(2) Vitamin A containing diet

d. Vitamin A trans-retinol (Eastman Kodak Co.)

4. Methods:

a. The most effective way of obtaining a vitamin A deficient animal is to remove the vitamin from the diet of pregnant animals and maintain the mothers and later the offspring on a vitamin free diet. This procedure will be used for evaluation against simply placing 4 week old mice on a vitamin A-free diet at the time of weaning.

b. Mice will be inoculated IT one time every 14 days for 6 to 12 times for MCA and for 10 to 15 times with BP. The number of inoculations will depend on the condition of the animals.

c. The literature indicates AHH induction requires vitamin A. We will include a group of animals maintained on the vitamin A free diet but supplemented with trans-retinol vitamin A 4 hours prior to IT inoculation with the chemical carcinogen.

d. In order to study the effects of vitamin A on AHH induction and to follow the immunological competence in these animals we will sacrifice 3 mice 48 hours after MCA or BP treatment. The lungs and livers will be used for AHH studies while the spleen will be used for cellular immunity studies. Appropriate controls will be included.

e. To study the influence of vitamin A deficiency on the histopathology of the animals, we will sacrifice 3 mice 13-14 days after chemical carcinogen treatment. Appropriate controls will be included. One set of tissues will be kept in the event we wish to pursue scanning electron microscopy at a later date.

f. Vitamin A deficiency will be established by assay of mouse sera or liver tissue.

g. Weight of the animals will be followed to demonstrate effects of avitaminosis.

h. Experimental design:

Group I Mice maintained on Vitamin A diet
- A Vehicle controls
- B Carcinogen treated

Group II Mice maintained on Vitamin A deficient diet
- A Vehicle controls
- B Carcinogenstreated

Group III Mice maintained on vitamine A deficient diet but given trans-retinol Vitamin A 4 hours prior to carcinogen treatment
- A. Vehicle controls
- B Carcinogen treated

Group IV Untreated controls on normal diet

5. References

Chan, P.C., Okamoto, T., Wynder, E.L. Possible Role of Riboflavin Deficiency in Epithelial Neoplasia. III. Induction of Microsomal Aryl Hydrocarbon Hydroxylase. J. Natl. Cancer Inst. 48: 1341-1345, 1972.

Cohen, B.E., Cohen, I.K. Vitamin A: Adjuvant and Steroid Antagonist in the Immune Response. J. of Immunology 111: 1376-1380, 1973.

Cone, M.V., Nettesheim, P. Effects of Vitamin A on 3-Methylcholanthrene-Induced Squamous Metaplasias and Early Tumors in the Respiratory Tract of Rats. J. Natl. Cancer Inst. 50: 1599-1606, 1973.

Crocker, R.R., Sanders. L.L. Influence of Vitamin A and 3,7-Dimethyl-2,6-octadienal (Citral) on the Effect of Benzo(a)pyrene on Hamster Trachea in Organ Culture. Cancer Research 30: 1312-1318, 1970.

Czygan, P., Greim, H., Garro, A., Schaffner, F., Popper, H. The Effect of Dietary Protein Deficiency on the Ability of Isolated Hepatic Microsomes to Alter the Mutagenicity of a Primary and a Secondary Carcinogen. Cancer Research 34: 119-123, 1974.

Davies, R.E. Effect of Vitamin A on 7,12-Dimethylbenz(a)anthracene-Induced Papillomas in Rhino Mouse Skin. Cancer Research 27: 237-241, 1967.

Harris, C.C., Sporn, M.B., Kaufman, D.G., Smith, J.M., Baker, M.S. Saffiotti, U. Acute Ultrastructural Effects of Benzo(a)pyrene and Ferric Oxide on the Hamster Tracheobronchial Epithelium. Cancer Research 31: 1977-1989, 1971.

Harris,C.C., Sporn, M.B., Kaufman, D.G., Smith, J.M., Jackson, F.E. Saffiotti, U. Histogenesis of Squamous Metaplasia in the Hamster Tracheal Epithelium Caused by Vitamin A Deficiency or Benzo(a)pyrene-Ferric Oxide. J. Natl. Cancer Inst. 48: 743-761, 1972.

Harris, C.C., Kaufman, D.G., Sporn, M.B., Saffiotti, U. Histogenesis of Squamous Metaplasia and Squamous Cell Carcinoma of the Respiratory Epithelium in an Animal Model. Cancer Chemother. Rep. 4: 43-54, 1973.

Hill, D.L., Shih, T.W. Vitamin A Compounds and Analogs as Inhibitors of Mixed-Function Oxidases that Metabolize Carcinogenic Polycyclic Hydrocarbons and Other Compounds. Cancer Research 34: 564-570, 1974.

Lasnitzki, I., Goodman, D.W. Inhibition of the Effects of Methylcholanthrene on Mouse Prostate in Organ Culture by Vitamin A and its Analogs. Cancer Research 34: 1564-1571, 1974.

Polliack, A., Levij, I.S. The Effect of Topical Vitamin A on Papillomas and Intraepithelial Carcinomas Induced in Hamster Cheek Pouches with 9,10-Dimethyl-1,2-benzanthracene. Cancer Research 29: 327-332, 1969.

Rogers, A.E., Sanchez, O., Feinsod, F.M., Newberne, P.M. Dietary Enhancement of Nitrosamine Carcinogenesis. Cancer Research 34: 96-99, 1974.

Rasmussen, R.E., Wang, I.Y., Crocker, T.T. Vitamin A-Induced Modification of Benzo-(a)pyrene Metabolism in Syrian Hamster Cell Cultures. J. Natl. Cancer Inst. 49: 693-700, 1972.

Saffiotti, U., Montesano, R., Sellakumar, A.R., Borg, S.A. Experimental Cancer of the Lung. Cancer 20: 857-864, 1967.

Seifter, E., Zisblatt, M., Levine, N., Rettura, G. Inhibitory Action of Vitamin A on a Murine Sarcoma. Life Sciences 13: 945-952, 1973.

Shamberger, R.J., Inhibitory Effect of Vitamin A on Carcinogenesis. J. Natl. Cancer Inst. 47: 667-673, 1971.

Smith, W.E., Hazdi, E., Miller, L. Carcinogenesis in Pulmonary Epithelia in Mice on Different Levels of Vitamin A. Environmental Research 5: 152-163, 1972.

B. EXPERIMENT CTR-39: Relationship between sensitivity to MCA-induced squamous cell carcinomas and inducibility of AHH.

1. Purpose: Results with CTR-5 yielded no clear-cut answers as to the relationship between genetically mediated levels of AHH and susceptibility to MCA tumors. The tumor response was just too low to make any comparisons. Consultations with Dr. P. Nettesheim and Mr. W. Blair have indicated that the particle size of the MCA may have been the problem. In this study, we intend to use the C3H/f Mai, the DBA/2J and various crosses between these strains to demonstrate the relationship between AHH inducibility and sensitivity to MCA-induced lung tumors.

2. Materials:

a. Mice
(1) C3H/f Mai, 100, ♂, ♀, 8-12 weeks old
(2) DBA/2J ,100, ♂,♀, 8.12 weeks old
(3) C3D2F1, 100, ♂, ♀, 8-12 weeks old
(4) C3D2F1 X D2, 100, ♂, ♀, (all diff. backcrosses)
(5) C3D2F1 X C3, 100, ♂, ♀, (all diff. backcrosses)
(6) C3D2F2, 100, ♂, ♀, (both F2s)

b. Chemicals

-MCA at 250μg/.02ml 2% gelatin

Materials for IT instillation and AHH assay.

3. Methods:

a. At 8-12 weeks of age, give 250μg/.02ml .2% gelatin to mice IT. Mice are to be treated with MCA six times in a twelve week period.

b. At 3 months post-treatment, check every other day for the external symptoms of lung tumors in mice.

c. When external symptoms (severe) are observed, mice will be induced with 80μg MCA/g body weight. and 24 hours later livers will be excised and stored in two pieces in two separate freezers. Gross and histopathological will be done.

d. Need to know precisely the incidence and latency period for each tumor of each group of animals.

e. Must check all mice very closely for external symptoms.

1003536369

f. At 8 months post-treatment with MCA, take all remaining animals off test: induce with MCA and freeze livers (2 parts)

g. Assay all samples in minimal number of days to provide best analysis of comparative AHH inducibility.

Date on test: 9/2/74

Date off test: about 3/20/75

C. EXPERIMENT CTR-40: Effect of TCDD on MCA carcinogenesis in DBA/2 mice.

1. Purpose: CTR-15, 16 and 17 suggested that TCDD, when given simultaneously with 150μg MCA, would enhance the tumorigenic effects of this dose of MCA. This study is designed to confirm that observation and to determine if MCA and TCDD, given simultaneously in the same vehicle can yield an even higher tumorigenic response.

2. Materials:

a. Mice
(1) 700 - DBA/2 ♀ - 6-8 weeks old.

b. Chemicals
(1) 150μg MCA/.05 ml trioctanoin
(2) 2.4μg TCDD plus 150μg MCA/.01 ml dioxane plus .04 ml trioctanoin
(3) .024μg TCDD plus 150μg MCA per 0.01 ml dioxane plus 0.04 ml trioctanoin
(4) 0.01 ml dioxane plus 0.04 ml trioctanoin

3. Methods:

a. Groups

	Days -2	0		# mice
(1)	none	MCA		30
(2)	Diox	MCA		50
(3)	none	Diox (IP) :MCA (SC)		50
(4)	none	Diox (SC) :MCA (SC)		70
(5)		TCDD (L) :MCA		100
(6)		TCDD (H) :MCA		100
(7)		TCDD (L) :MCA	together	100
(8)		TCDD (H) :MCA	together	100
(9)	TCDD (H)	MCA		100
		Total		700

b. 2 days after TCDD, randomly take two mice per group and freeze the liver for later AHH testing.

c. Making up TCDD:MCA solutions
(1) make up 3.75 mg MCA/ml trioc
(2) make up 240 μg TCDD/ml dioxane

1003536371

(3) mix 4 parts MCA with 1 part TCDD
∴ 300μg MCA/ml
and 48μg TCDD/ml
-if giving 0.05 ml/mouse
then: 150μg MCA
and 2.4μg TCDD

(4) Control vehicle = 4 parts trioc plus
1 part dioxane

(5) For low TCDD dose, use a 1:100 dilution of the 240μg TCDD/ml solution in dioxane and add 1 part of this dilution to 4 parts MCA (in trioc).

Date on test: 8/21/74
Date off test: 3/21/75

1003536372

1003536373

Suppl Studies

A. EXPERIMENT CTR-41 : Use of inhibitors and inducers of AHH and their effect on MCA induced lung tumors in C3H, B6 and D2 mice.

1. Purpose: Of prime importance is the use of material to epigenetically alter the susceptibility and resistence to chemical carcinogens. Our model system will consist of intratracheal instillation of 250μg MCA every other week for 12 weeks. Inducers and/or inhibitors will be either given previously or simultaneously with the MCA and the role of these chemicals on pulmonary AHH and pulmonary tumors will be evaluated.

2. Materials:

a. Mice
(1) C3H/f Mai, ♀, 8-10 weeks old
(2) DBA/2 Cum, ♀, 8-10 weeks old
(3) C57Bl/6 Cum, ♀, 8-10 weeks old

b. Carcinogen: MCA, 250 μg/.02 ml 0.2% gelatin

c. Chemicals:
(1) TCDD - a potent inducer of AHH
(2) Phenobarbital - an inducer of constitutive AHH
(3) 7,8-benzoflavone - an inhibitor of AHH
(4) 5,6-benzoflavone - an inducer of AHH
(5) vitamin A - an inducer of AHH
(6) SKR-525A - an inhibitor of constitutive AHH

3. Methods:

a. The toxic effects of each chemical will be established by determination of LD_{50-20} using 10 mice per chemical (B6 mice).

b. The effects on pulmonary AHH will be established.

c. A maximum of 3 or 4 of the above chemicals will be given 24 hrs prior to MCA treatment and simultaneously with MCA and held for presence of lung tumors.

d. 3-4 months after MCA 10 animals per group will be sacrificed and lungs sent for pathologic and histologic analysis.

e. If the incidence of tumors approximates 50% in the controls then all will be sacrificed.

f. If no tumors, wait till 5 months post-treatment and repeat pathologic tests.

g. Observe # tumors per treated for each group.

1003536374

B. EXPERIMENT CTR-42 : Determination of the classes of chemical carcinogens and the types of tumors initiated by these carcinogens which are influenced by changes in AHH inducibility.

1. Purpose: In the cross B6D2F1 X D2, AHH inducibility segregates as a single gene yielding 50% of the progeny AHH inducible. This is a perfect population to determine the role of AHH inducibility in cancers induced by classes of carcinogens other than polycyclic aromatic hydrocarbons. Perhaps, in this way, it can be shown that some risk also exists for the non-inducible (or low-inducible) populations.

2. Materials:

   a. Mice
      B6D2F1 X D2, ♀ and ♂, 4-6 weeks old

   b. Chemicals
      (1) MCA 250µg/0.02 ml 0.2% gelatin
      (2) 2-acetylaminofluorene (AAF)
      (3) N-nitrosodimethylamine (DMN)
      (4) urethane
      (5) N,N-dimethyl-4-aminoazobenzene (DAB)

   c. Vehicle
      0.2% gelatin in sterile saline

3. Methods:

   a. Precheck all mice for AHH inducibility using zoxazolamine-induced sleeping time.
   b. Determine LD_{10-20} for each chemical using 10 mice (B6).
   c. Give this dose IT to 100 backcross mice.
   d. At 6 months take 10 mice off test and do complete autopsy. Check kidney, liver, bladder, as well as lung, for tumors.
   e. Take 10 animals per month off test and if 70-80% show signs of tumors take rest of animals off test.
   f. Do complete autopsy.

1003536375

Schedule

1003536376

Projected Initiation and Completion Dates of Proposed Experiments

On Going:

CTR-1: SC Assay IRI CSC fractions
CTR-1A: SC Assay IAI CSC fractions
CTR-1B: SC Assay whole CSC from Dr. Gori
CTR-2: IP carcinogenesis of nitrosamines
CTR-2A: Repeat of CTR-2
CTR-3: IT injection of MCA in C3H/f, C57BL/6, C57BL and hybrid mice
CTR-3A: Repeat of CTR-3 in C3H/f mice using 2 vehicles, 2 schedules and 2 doses
CTR-3B: Repeat of CTR-3A in C57BL/6 mice
CTR-3C: Repeat of CTR-3 in C3H/f ♀ mice using 3 doses for 6-12 weeks
CTR-3D: Repeat of CTR-3C in C3H/f ♂ mice
CTR-4: SC-MCA carcinogenesis in C57BL, C3H/f and hybrid mice
CTR-5: IT injection of MCA in C57BL/6, DBA/2 and hybrid mice
CTR-15:
CTR-16: TCDD effects on MCA carcinogenesis
CTR-17:
CTR-18: IT DEN injection in DBA/2 mice
CTR-18A: Wax pellet DMN lung carcinogenesis

Proposed:

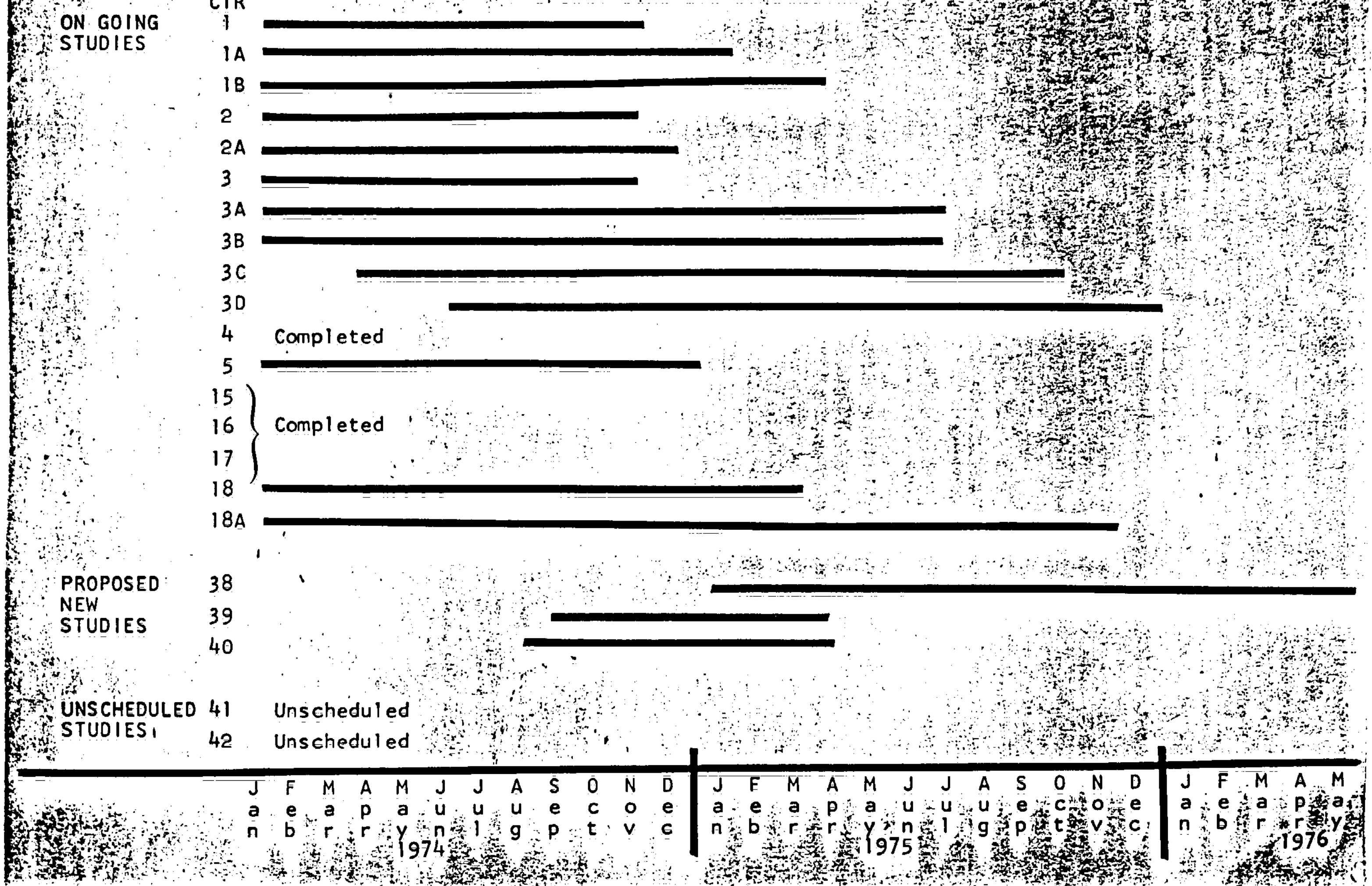
CTR-38: Vitamin A effects on lung carcinogenesis, AHH and immunocompetence
CTR-39: Repeat of CTR-5 using C3H, DBA and hybrid mice
CTR-40: Effect of TCDD on MCA Carcinogenesis in DBA/2 Mice

Supplementary Unscheduled Studies:

CTR-41: Use of Inhibitors and Inducers of AHH and their Effect on MCA
CTR-42: Other Chemicals in Lung Carcinogenesis.

1003536377

Proposed Schedule of Studies



Budget

1003536379

BUDGET

The budget proposed for the contract year 1975 reflects the increases incurred by the massive inflation we are now experiencing and expect to continue during the contract year. This is reflected not only in personnel salaries but other direct costs.

Our cost for plastic cages, animal food and apples as a source of water, have tripled during the past 18 months. We feel this will probably triple again during the next 18 months therefore we are attempting to control this cost by the conversion to permanent cages with automatic watering. The cost of other supplies and mice and the freight on their shipment to us has also increased dramatically.

This budget also reflects the establishing of an in-house computer programing capability and the necessity of renting computer time. We have in the past been able to take advantage of of services available on other contracts.

Due to the long term holding of the mice for chemical carcinogenesis experiments it has been necessary to expand our animal holding facilities and add an additional animal caretaker. The additional facilities will be equipped with shower facilities to meet new government regulations for handling certain chemical carcinogens being used in the CTR Program.

The personnel and positions listed in the budget differ somewhat from that in last year's budget. We have moved personnel and their services within the CTR Contracts in order to establish better budget accountability. The personnel budgets have not however, reflected a dollar change on this basis. The histological service provided in this contract in 1974 Budget has been transferred to another CTR contract instead of splitting it between contracts. This makes for easier accounting procedures. We will provide you with a personnel schedule reflecting the labor distribution between the various CTR-MA contracts.

Renewal for twelve months, Jan. 1, 1975 through Dec 31, 1975

A.	Direct Labor (Schedule A)	$ 52,913.00
B.	Overhead (115% of A)	60,850.00
C.	Other Direct Cost (Schedule B)	23,500.00
D.	Travel	500.00
E.	General and Administrative (16% of $137,763.)	$22,042.00
F.	Total Cost	$ 159,805.00
G.	Fixed Fee	17,755.00
H.	Total Before Equipment	$ 177,560.00
I.	Equipment (Schedule C)	4,000.00
J.	Total Price	$ 181,560.00

Schedule A: Direct Labor

Personnel - Position	Time on Project	Total Hrs.	Rate/ Hr.	Amount $
C. E. Whitmire, Ph.D. Co-Project Director	10%	193	13.51	2,608.00
C. F. Demoise, Ph.D. Assoc. Project Director	50%	482	8.18	7,877.00
M. Haven, M.S. Computer Programer	40%	770	10.58	8,147.00
S. Gosnell, Technician	100%	1926	3.97	7,646.00
Vacancy, Technician	100%	1926	3.97	7,646.00
A. Zuna, Animal Caretaker	100%	1926	3.28	6,317.00
Vacancy, Animal Caretaker	100%	1926	2.75	5,297.00
A. Saborit, Lab. Aide	50%	963	3.17	3,053.00
D. Powers, Adm. Assist.	15%	289	4.21	1,217.00
P. Gradwell, Res. Clerk	50%	963	3.31	3,188.00
B. Ross, Key Punch Oper.	40%	770	3.00	2,310.00
		12,133		$51,372.00
6% Merit Raise (3% for 6 mo.)				1,541.00
Total Direct Labor				$52,913.00

1003536382

Schedule B: Other Direct Costs

Cages, Mouse Food	$11,000.00
General Supplies	6,000.00
Mice	2,500.00
Computer Time	4,000.00
Total Other Direct Costs	$23,500.00

Schedule C: Equipment

During the next 12 months we will convert from disposable cages to permanent cages with automatic watering to avoid the continued increase in cost of plastic cages and apples as a a water source. The cost of the cages listed above included the cost of plastic cages and the conversion to permanent cages. In addition to this cost we will incur the cost of installing automatic watering to existing racks. Depending on the size of the cage rack the cost varies from $400 - 530/rack. We anticipate converting a minimum of 10 racks to automatic watering for this contract during the 1975 contract year.

$4,000.00

1003536385

Publications

VII. PUBLICATIONS - 1974

Whitmire, C. E., Demoise, C. F., Kouri, R. E. The Role of the Host in the Development of In Vivo Models for Carcinogenesis Studies. In: Symposium on Experimental Respiratory Carcinogenesis and Bioassays (ed. J.F. PARKS & E. KARBE) Springer-Verlag (in press) 1974

Demoise, C.F., Kouri, R.E., and Whitmire, C.E. Cell-Mediated Immunity After Intratracheal Exposure to 3-Methylcholanthrene, and its Relationship to Tumor Transplant Growth in C3H/fMai Mice. In: Symposium on Experimental Respiratory Carcinogenesis and Bioassays (ed. J.F. PARKS & E. KARBE) Springer-Verlag (in press) 1974.

Kouri, R.E., Demoise, C.F., Whitmire, C.E. The Significance of the Aryl Hydrocarbon Hydroxylase Enzyme Systems in the Selection of Model Systems for Respiratory Carcinogenesis. In: Symposium on Experimental Respiratory Carcinogenesis and Bioassays (ed. J.F. PARKS & E. KARBE) Springer-Verlag (in press) 1974

Kouri, R.E., Ratrie III, H., Whitmire, C.E. Genetic Control of Susceptibility to 3-Methylcholanthrene-Induced Subcutaneous Sarcomas. Int. J. Cancer 13: 714-720, 1974

Kouri, R.E., Kiefer, R., Zimmerman, E.M. Hydrocarbon-Metabolizing Activity of Various Mammalian Cells in Culture. In Vitro (in press) 1974

Kouri, R.E., Ratrie III, H.,Atlas, S.A., Niwa, A., Nebert, D.W. Aryl Hydrocarbon Hydroxylase Induction in Human Lymphocyte Cultures by 2,3,7,8-Tetrachlorodibenzo-p-Dioxin. Life Sciences (in press) 1974

Benedict, W.F., Rucker, N., Mark, C., Kouri, R.E. Correlation Between the Balance of Specific Chromosomes and the Expression of Malignancy in Hamster Cells. J. Natl. Cancer Inst. (in press) 1974

Kouri, R.E., Rude, T.H., Thomas, P.E., Whitmire, C.E. Studies on Pulmonary Aryl Hydrocarbon Hydroxylase in Inbred Strains of Mice. (submitted) 1974

Kouri, R.E., Kurtz, S.A., Price, P.J., Benedict, W.F. Studies on the ara-C-Induced Malignant Transformation of Hamster and Rat Cells in Culture. (submitted) 1974

Kouri, R.E. Genetic Control of Susceptibility to Cancer Induced by 3-Methylcholanthrene (MCA) Proceedings of the XI International Cancer Congress, October 1974

1003536387

VIRUS IN CHEMICAL
CARCINOGENESIS

LEVY - U.C.M.C.

1003536388

Material on Jay Levy, M. D., University of California School of Medicine, will be found in Supplement under No. 1011.

MICROBIOLOGICAL

MICROBIOLOGICAL ASSOCIATES
Division of DYNASCIENCES Corporation
4733 Bethesda Avenue / Bethesda, Maryland 20014 / (301) 654-3400

September 13, 1974

Dr. John Kreisher
Council for Tobacco Research
110 East 59th Street
New York, N.Y. 10022

Dear John:

Enclosed is a copy of my budget for providing animals for Dr. Jay Levy. The assistance alluded to in the grant proposal made this additional budget mandatory. Our laboratory will do the following:

a) Provide about 850 animals from crosses involving the 129/J and NZB strains of mice. Included will be about 50 ♀ and ♂ mice from the following crosses: parent, F_1, F_1 x 129/J, 129/J x F_1, F_1 x NZB, NZB x F_1, and F_2. We need to breed about 650 mice and we need 250♀ and 50♂ to generate this number of animals. Therefore, about 1,150 animals will be housed for the year.

b) Partial splenectomy will be performed on most of these mice and shipped to Dr. Levy for virus isolation.

c) Sera will be taken at predetermined intervals and tested for antibody titers against the xenotropic virus by Dr. Levy.

d) Animals will be treated with 500 μg MCA and palpated weekly for subsequent tumor formation.

e) The role of the xenotropic, ecotropic and anti-sera against these viruses in MCA-induced tumorigenesis will be determined.

f) AHH assays on a representative number of animals will also be done.

1003536391

I hope this budget meets with the approval of both Dr. Levy and the CTR.

Also enclosed are 10 copies of a progress report on the human AHH contract.

Respectfully yours,

Dick

Richard E. Kouri, Ph.D.
Department Head
Dept. of Biochemical Oncology

Enclosures
mrl

1003536392

Tentative budget for collaborative project with Dr. J. Levy, University of California, San Francisco Medical Center, San Francisco, California. (January 1, 1975 - December 31, 1975)

A.	Total Direct Labor (Schedule A)	$10,574.00
B.	Overhead (115% of A)	12,160.00
C.	Other direct costs (Schedule B)	8,834.00
D.	Total (A-C)	$31,568.00
E.	General and Administration (16% of D	5,051.00
F.	Total	$36,619.00
G.	Fixed Fee (10%)	4,066.00
H.	Total Costs	$40,685.00

Schedule A

Direct Labor

Name and Position	Time on Project	Total Hrs	$/hrs	Total
R. E. Kouri, Ph.D. Project Director	5%	96	NC	NC
T. Rude, Technician	50%	963	4.46	$4,295.
Vacancy, Lab Aide	100%	1926	3.10	5,971.
		2985		$10,266.
3% Anticipated Merit Increases				308.
Total				$10,574.

1003536394

Schedule B

Other Direct Costs

Materials

Animals (250-129/J)	$500.00	
Feed and Bedding (total 1150 animals)	1,050.00	
Chemicals	150.00	
Total Materials		$1,700.00

Expendable Supplies

Disposable Cages Synginges, needles, tubes	$6,100.00	
Stainless steel lids	750.00	
Total Supplies		6,850.00
Shipping		284.00
Total Other Direct Costs		$8,834.00

1003536395

Tentative budget for collaborative project with Dr. J. Levy, University of California, San Francisco Medical Center, San Francisco, California. (January 1, 1975 - December 31, 1975)

A.	Total Direct Labor (Schedule A)	$10,574.00
B.	Overhead (115% of A)	12,160.00
C.	Other direct costs (Schedule B)	8,834.00
D.	Total (A-C)	$31,568.00
E.	General and Administration (16% of D	5,051.00
F.	Total	$36,619.00
G.	Fixed Fee (10%)	4,066.00
H.	Total Costs	$40,685.00

1003536396

Schedule A

Direct Labor

Name and Position	Time on Project	Total Hrs	$/hrs	Total
R. E. Kouri, Ph.D. Project Director	5%	96	NC	NC
T. Rude, Technician	50%	963	4.46	$4,295.
Vacancy, Lab Aide	100%	1926	3.10	5,971.
		2985		$10,266.
3% Anticipated Merit Increases				308.
Total				$10,574.

1003536397

Schedule B

Other Direct Costs

Materials		
Animals (250-129/J)	$500.00	
Feed and Bedding (total 1150 animals)	1,050.00	
Chemicals	150.00	
Total Materials		$1,700.00
Expendable Supplies		
Disposable Cages SyngInges, needles, tubes	$6,100.00	
Stainless steel lids	750.00	
Total Supplies		6,850.00
Shipping		284.00
Total Other Direct Costs		$8,834.00

1003536398

1003536399

ENGINEERING

1003536400

OAK RIDGE

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

September 27, 1974

To: W. U. Gardner, Scientific Advisory Board

Subject: Engineers Costs - Oak Ridge

During the past year extensive troubleshooting modifications, adaptations, etc. have been made to the Lorillard Machine. This has required extensive engineering manpower not anticipated in the original protocol. To date this has required shifting priorities in the program.

An engineering budget at $15,000, to be drawn down as required, through billing, is necessary to integrate timing circuits, animal holders, etc., and to continue troubleshooting when required for the Lorillard and Process & Instruments machines over the next six months.

J.H.K.

JHK:wg

1003536401

1003536402

PROCESS & INSTR.

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

September 27, 1974

To: W. U. Gardner, Scientific Advisory Board

From: J. H. Kreisher

Subject: Engineering Costs - Process & Instruments

Considerable engineering costs will be incurred during the next year to provide prototypical animal holders, modified timing circuits, new smoke vent accessories, etc. for Oak Ridge. These will require a draw down account to assure continuity.

A request for $25,000 allocation, to carry out such engineering activities is requested.

J.H.K.

JHK:wg

1003536403

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

September 27, 1974

To: W. T. Hoyt, W. U. Gardner, Robert C. Hockett

From: J. H. Kreisher

Subject: Authorization for commercial development of Walton Horizontal Smoke Exposure Machine

The attached request for consideration of release of current prototypical smoking machine design has been received.

The machine is being successfully used by a number of investigators, and the design for intermittent smoking appears to be about as "final" as any design ever will be.

Chemical studies of smoke constituents, mixing characteristics, and small animal (mouse) dosimetry are well underway or will be initiated within the next few months. The first backup material concerning smoke composition is being prepared for publication; and additional studies, as completed, will be published to provide additional direction to non-familiar investigators. These findings are also being incorporated into a Manual. Drawings are being made for a final "Manual", which is currently being rewritten.

Animal holder studies are progressing well. The mouse and rat adapt seemingly well in the newest holder configurations. Stress (serum corticosteroid level and brain protein production) studies are continuing, and data for adaptation to specific holder types with and without chronic intermittent smoke exposure over a prolonged (two month) period will be available soon.

It is estimated that, once permission is given to build machines commercially, the earliest that these could be available for sale in any numbers would be one year from now, due to delays in parts delivery, etc.

The advanced status of prototype development and the satisfactory performance characteristics (over one year in continuous 24 hr/dy operation without breakdown), make consideration of a release request for commercial production reasonable at this time.

J.H.K.

JHK:WG

1003536404

p&i PROCESS & INSTRUMENTS CORPORATION
1943 Broadway, Brooklyn, N.Y. 11207, Tel. 212 - 452-8380

September 24, 1974

Dr. John Kreisher
The Council for Tobacco Research-U.S.A.,Inc.
110 East 59th Street
New York,N.Y. 10022

Dear Doctor Kreisher:

We have received numerous requests, many of them referred to us by the Council for Tobacco Research-U.S.A.,Inc., for price and delivery information on the Walton Horizontal Smoke Exposure Machine. While this machine was under development for the Council, we did not respond to these requests. Now, however, since the current version of the machine has proven satisfactory in extensive field tests, we would like to make it generally available to interested groups.

Therefore we are hereby applying to the Council for Tobacco Research-U.S.A.,Inc. for permission to manufacture and market the Walton machine for sale to the general scientific public.

We trust that the Council will grant our request and thereby permit us to make this useful device available to research investigators.

Sincerely yours,
PROCESS & INSTRUMENTS CORPORATION

Joseph Greenspan

Joseph Greenspan,Ph.D.
Director

JG/es

1003536405

FRACTIONATION

1003536406

PATEL - MELOY

1003536407

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

September 27, 1974

To: W. U. Gardner, Scientific Advisory Board

From: J. H. Kreisher

Subject: Smoke Fractionation: Meloy Laboratories

The fractionation of smoke by the Stedman fractionation procedure (Swain, A. P., Cooper, J. E. and Stedman, R. L., Large-Scale Fractionation of Cigarette Smoke Condensate for Chemical and Biologic Investigations. Cancer Research 29 579-583 (1959)) have provided fractions used by CTR contractors and grantees in such studies as co-carcinogenesis, mutagenesis (Salmonella), *in vitro* transformation, viral induction, and to initiate studies of DNA repair inhibition. It is estimated that four fractionations of this material will be used over the next year to provide reasonably fresh condensate fractions. The cost for four such fractionations would approximate $14,000. A budgetry allocation approval for that amount to assure continuity in the research programs underway is requested.

J.H.K.

JHK:wg

1003536408

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

September 27, 1974

To: W. U. Gardner, Scientific Advisory Board

From: J. H. Kreisher

Subject: Subfractionation of Biologically Active Smoke Condensate Fractions

During the past year the crude fractionation of smoke condensates and crude condensates alone have shown significant differences in the following areas:

1. AHH induction
2. Mutagenesis
3. In vitro transformation
4. Subcutaneous in vivo co-carcinogensis

Requests have been received to provide subfractionated or fractionated material via column chromotography methods (to avoid oxidation artefacts). Since no methods are available for this fractionation procedures a "best effort" program would be the only possible approach. Staff has requested an estimate, based on knowledge of correct technology and projected costs from Oak Ridge National Laboratory to devise an appropriate subfractionation schematic and proceed to provide fractions of defined constituents which would be tested for biological significance in the currently available test systems.

This is a complicated undertaking, requiring sophisticated analytical and column fractionation methodology far in excess of any efforts to date.

If such studies were to be undertaken an estimated $150-175,000 per annum would be required to undertake these studies during the next year.

J.H.K.

JHK:wg

1003536409

OAK RIDGE

1003536410

1003536411